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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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**(54) Title:** TUMOR NECROSIS FACTOR RECEPTOR-DERIVED PEPTIDE ANALOGUES**(57) Abstract**

The present invention relates to peptides and peptide analogues designed from a binding loop of a member of the tumor necrosis factor receptor (TNF-R) superfamily. In particular, it relates to cyclic peptides and peptide analogues designed from a binding loop of TNF-R which inhibit TNF binding to its cellular receptors, and methods of making and using such compounds to inhibit the biological activities of TNF.

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## TUMOR NECROSIS FACTOR RECEPTOR-DERIVED PEPTIDE ANALOGUES

1. INTRODUCTION

5       The present invention relates to peptides and peptide analogues designed from a binding loop of a member of the tumor necrosis factor receptor (TNF-R) superfamily, which is involved in binding interactions with its ligand. In particular, it relates to cyclic peptides and peptide  
10 analogues designed from three specific binding loops in domains 2 and 3 of TNF-R which inhibit tumor necrosis factor (TNF) binding to its cellular receptors, methods of designing similar peptides and peptide analogues, and methods of using such compounds to inhibit the biological activities of TNF,  
15 thereby antagonizing its undesirable clinical effects.

2. BACKGROUND OF THE INVENTION2.1. TUMOR NECROSIS FACTOR AND ITS PATHOPHYSIOLOGY

20       TNF was originally discovered as a molecule which caused hemorrhagic necrosis of mouse tumors (Carswell et al., 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3666). A second line of investigation of a serum protein known as "cachectin", thought to be responsible for the condition of cachexia, led  
25 to the eventual discovery that cachectin was identical to TNF (Beutler et al., 1989, Annu. Rev. Immunol. 7:625). TNF has now been established as a broadly active inflammatory mediator involved in diverse clinical conditions.

      TNF/cachectin was renamed as TNF- $\alpha$ , and a structurally  
30 and functionally related protein previously known as lymphotoxin (LT) was referred as TNF- $\beta$  (Vassalli, 1992, Annu. Rev. Immunol. 10:411). Both molecules are active as homotrimers and mediate similar biological effects by binding to the same two cellular receptors of 55kD (p55 or complex I)  
35 and 75kD (p75) molecular weight (Smith et al., 1990, Science 248:1019; Schall et al., 1990, Cell 61:361). An LT heterotrimer was later discovered, which engaged a third receptor known as TNF-R related protein (rp), but it is not

capable of binding to TNF-R p55 and p75 (Browning et al., 1993, Cell 72:847). This LT has been named as LT- $\beta$ , and the LT homotrimer (or TNF- $\beta$ ) is also referred to as LT- $\alpha$ .

Structural comparison of the three TNF-R with several other  
5 cell surface receptors has resulted in the classification of these receptors into the TNF-R superfamily (Gruss and Dower, 1995, Cytokines and Mol. Ther. 1:75).

TNF- $\alpha$  is a 17 kD molecular weight protein produced by several cell types, particularly activated macrophages.

10 Since TNF-R is expressed by numerous cell populations, TNF induces a wide variety of cellular responses, many of which result in deleterious consequences. For example, TNF induces cachexia which is a condition resulting from loss of fat and whole body protein depletion, accompanied by insufficient  
15 food intake due to anorexia. Cachexia is commonly seen in cancer patients, and it has also been observed in patients with acquired immunodeficiency syndrome (AIDS).

In addition, injection of high doses of TNF in animals produces most of the symptoms of septic shock. TNF has also  
20 been shown to play a role in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, hypersensitivity, immune complex diseases and graft versus host disease as well as transplantation rejection. The involvement of TNF has even been implicated in malaria and  
25 lung fibrosis.

## 2.2. TREATMENT OF TNF-ASSOCIATED DISORDERS

Methods for neutralizing the adverse effects of TNF have focused on the use of anti-TNF antibodies and soluble TNF-R.  
30 In animal models, treatment of TNF-associated inflammatory disorders with antibodies specific for TNF has shown therapeutic efficacy (Williams et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:9784; Baker et al., 1994, Eur. J. Immunol. 24:2040; Suitters et al., 1994, J. Exp. Med.  
35 179:849). Chimeric forms of anti-TNF antibodies have been constructed for use in human clinical trials (Lorenz et al., 1996, J. Immunol. 156:1646; Walker et al., 1996, J. Infect.

Dis. 174:63; Tak et al., 1996, Arthritis Rheumat. 39:1077). Additionally, soluble TNF-R fusion proteins have been introduced as TNF-antagonists in human patients (Peppel et al., 1991, J. Exp. Med. 174:1483; Williams et al., 1995, 5 Immunol. 84:433; Baumgartner et al., 1996, Arthritis Rheumat. 39(Suppl.) S74).

While the aforementioned approaches have shown some effectiveness in certain disease conditions, anti-TNF antibodies and soluble TNF-R both suffer from common 10 limitations of macromolecules such as poor bioavailability and stability, induction of immune reactions and ineffective tissue penetration. Thus, there remains a need for improved therapeutic compounds for antagonizing the undesirable effects of TNF.

15

### 3. SUMMARY OF THE INVENTION

The present invention relates to peptides and peptide analogues designed from a binding loop of a TNF-R superfamily member. In particular, it relates to peptides and peptide 20 analogues designed from three binding loops of TNF-R. More specifically, the invention relates to peptides and peptide analogues which interfere with the binding interactions between TNF and TNF-R, methods of designing additional peptides and peptide analogues exhibiting inhibitory 25 activities, and methods of using such compounds and pharmaceutical compositions thereof to antagonize the undesirable biological activities of TNF *in vivo*, as well as methods of using the compounds to detect the presence of TNF in a sample and for inhibiting TNF activities *in vitro*.

30 The invention is based, in part, on the Applicants' discovery that peptides designed from three binding loops of TNF-R p55 inhibited TNF- $\alpha$  binding to TNF-R. Such peptides were generated on the basis of the amino acid sequences that form the specific binding loops. Cys residues were added to 35 the peptides to enable their cyclization by formation of disulfide bridges, and hydrophobic aromatic residues were added to their termini to enhance structural stability.

Among the peptides that competitively inhibited the binding of TNF- $\alpha$  to its cognate receptor, peptides designed from loop 1 of domain 3 of TNF-R were the most effective.

Generally, a compound of the present invention is a  
 5 cyclic peptide or peptide analogue which is modified at its termini with hydrophobic moieties. In embodiments wherein the compound is a peptide, the peptide corresponds in primary sequence to a binding loop of a member of the TNF-R superfamily or a portion thereof. In a preferred embodiment,  
 10 the peptide corresponds in primary sequence to a binding loop of TNF-R p55 or a portion thereof. In certain embodiments, one or more amino acid residues within the peptide are substituted with other amino acid residues. Typically, such amino acid substitutions are conservative, i.e., the amino  
 15 acid residues are replaced with other amino acid residues having similar physical and/or chemical properties. In embodiments wherein the compound is a peptide analogue, the analogue is obtained by replacing at least one amide linkage in the peptide with a substituted amide or an isostere of  
 20 amide.

In an illustrative embodiment, a compound of the invention has the following formula:



wherein:

AC is a peptide of 3-18 amino acid residues which  
 30 corresponds in primary sequence to a binding loop of a TNF-R superfamily member, and which may optionally contain one or more conservative amino acid substitutions, or an analogue thereof wherein at least one amide linkage is replaced with a  
 35 substituted amide or an isostere of amide;

AB<sub>1</sub> is a moiety having a first functional group capable of forming a covalent linkage with one terminus of



AC, a second functional group capable of forming a covalent linkage with AB<sub>2</sub> and a third functional group capable of forming a covalent linkage with AA<sub>1</sub>;

- 5 AB<sub>2</sub> is a moiety having a first functional group capable of forming a covalent linkage with the second terminus of AC, a second functional group capable of forming a covalent linkage with AB<sub>1</sub> and a third functional group capable of forming a covalent  
10 linkage with AA<sub>2</sub>;  
AA<sub>1</sub> is a moiety having hydrophobic properties and a functional group capable of forming a covalent linkage with the third functional group of AB<sub>1</sub>;  
AA<sub>2</sub> is a moiety having hydrophobic properties and a  
15 functional group capable of forming a covalent linkage with the third functional group of AB<sub>2</sub>;  
"=" is a covalent linkage; and  
"≡" is a covalent linkage.

- 20 In a preferred embodiment of the compounds of formula (I) AC is a peptide which corresponds in primary sequence to a binding loop of TNF-R p55 and which optionally may contain one or more conservative amino acid substitutions, or an analogue thereof. In a particularly preferred embodiment,  
25 the peptides and peptide analogues specifically inhibit the binding of TNF to its cellular receptors. Therefore, such compounds are useful in antagonizing the undesirable biological activities of TNF. In that connection, a wide variety of specific uses are encompassed by the invention,  
30 including but not limited to, treatment of TNF-associated pathological conditions such as acute and chronic inflammatory responses, septic shock, cachexia, autoimmunity, graft-versus-host disease, skin allergic reactions, immune complex disease, transplantation rejection and malaria.

35

#### 4. BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1. Sequence alignment of amino acids in certain extracellular Cys-rich domains of TNF-R superfamily members: TNF-R p55 (SEQ ID NO:1), TNF-R p75 (SEQ ID NO:2), TNF-R-rp (SEQ ID NO:3), NGF-R p75 (SEQ ID NO:4), CD27 (SEQ ID NO:5), CD30 (proximal) (SEQ ID NO:6), CD30 (distal) (SEQ ID NO:7), CD40 (SEQ ID NO:8), Fas antigen (SEQ ID NO:9), OX40 (SEQ ID NO:10), and 4-IBB (SEQ ID NO:11).
- Figure 2. Inhibition of radiolabeled-TNF- $\alpha$  interaction with TNF-R. In a competitive radioreceptor assay,  $^{125}\text{I}$ -labelled TNF was preincubated with a peptide followed by binding to immobilized TNF-R. The peptides were compared at 25  $\mu\text{M}$  for their ability to inhibit the binding of TNF- $\alpha$  (10 nM) to 1nM of TNF-R-IgG chimeric protein.
- Figure 3. Inhibition of radiolabeled-TNF- $\alpha$  interaction with TNF-R in a dose-response assay. In a competitive radioreceptor assay, TNF- $\alpha$  binding to immobilized TNF-R-chimeric protein was inhibited in the presence of several WP9 series peptides in a defined dose range. The results represent the means and standard deviations from three independent experiments.
- Figure 4. Size exclusion HPLC profiles of exocyclic peptides. Each peptide (4mg) was eluted from Protein-Pak 60 column with 0.1M phosphate buffer at pH 7.0 at a flow rate of 1 ml/min. and their UV was monitored at 214 nm.
- Figure 5. FAC analysis of TNF- $\alpha$  binding to cellular receptors in the presence of WP9QY peptide. 1=U937 cells stained with fluorescein-

conjugated secondary antibody. 2=U937 cells stained with an anti-TNF receptor antibody (htr-9) and secondary antibody. 3=U937 cells stained with anti-TNF-R antibody in the presence of TNF- $\alpha$  and secondary antibody. 4=U937 cells stained with an anti-TNF receptor antibody (htr-9) and TNF- $\alpha$  preincubated with 250 $\mu$ M of WP9QY peptide.

Figure 6. FAC analysis of TNF- $\alpha$  binding to cellular receptors in the presence of one of three peptides derived from TNF-R. TNF- $\alpha$  binding to U937 cells were inhibited by each peptide in a dose-dependent manner. —●— = WP9QY peptide; - --□-- = WP9Q peptide; ---▲--- = WP5JY peptide. The results represent the means from two independent experiments.

Figure 7. Inhibition of TNF- $\alpha$ -induced cytolysis of L929 cells by peptides derived from TNF-R. Absorbance with 1  $\mu$ g/ml of ACT-D alone and with ACT-D plus 50 pg/ml of TNF- $\alpha$  are referred to as 100% survival and 100% cytotoxicity, respectively. The results indicate the means and standard deviations from three independent experiments.

Figure 8A & B Inhibition of TNF- $\alpha$ -induced insulin resistance by WP9QY peptide. Differentiated 3T3-L1 cells were stimulated with insulin, and Tyr phosphorylated IRS-1 was assayed. Certain samples were also treated with TNF- $\alpha$  which inhibited Tyr phosphorylation of IRS-1. Preincubation of TNF- $\alpha$  with WP9QY peptide restored Tyr phosphorylation of IRS-1 in response to insulin. Figure 8A shows the results of Western blot with an anti-

phosphorylated Tyr antibody after immunoprecipitation with an anti-IRS-1 antiserum. Figure 8B shows the results of Western blot following immunoprecipitation with the same anti-IRS-1 antiserum.

Figure 9. Three dimensional structural view of the binding sites in TNF-R for TNF- $\alpha$ . Monomeric TNF- $\alpha$  is shown on the right and monomeric TNF-R is shown on the left with the binding site in loop 1 of domain 3 shown in darkened color.

#### 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to cyclic peptides and peptide analogues which inhibit the binding interactions between a TNF-R superfamily member and its ligand. Although the specific procedures and methods described herein are exemplified using several specific peptides derived from TNF-R p55, they are merely illustrative for the practice of the invention. Analogous procedures and techniques, as well as functionally equivalent peptides and peptide analogues, as will be apparent to those of skill in the art based on the detailed disclosure provided herein are also encompassed by the invention.

As used herein, the following terms shall have the following meanings:

"Alkyl:" refers to a saturated branched, straight chain or cyclic hydrocarbon group. Typical alkyl groups include, but are not limited to, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, isopentyl, hexyl, and the like. In preferred embodiments, the alkyl groups are (C<sub>1</sub>-C<sub>6</sub>) alkyl, with (C<sub>1</sub>-C<sub>3</sub>) being particularly preferred.

"Substituted Alkyl:" refers to an alkyl group wherein one or more hydrogen atoms are each independently replaced with other substituents.

"Alkenyl:" refers to an unsaturated branched, straight chain or cyclic hydrocarbon group having at least one carbon-carbon double bond. The group may be in either the *cis* or *trans* conformation about the double bond(s). Typical alkenyl groups include, but are not limited to, ethenyl, propenyl, isopropenyl, butenyl, isobutenyl, *tert*-butenyl, pentenyl, hexenyl and the like. In preferred embodiments, the alkenyl group is (C<sub>1</sub>-C<sub>6</sub>) alkenyl, with (C<sub>1</sub>-C<sub>3</sub>) being particularly preferred.

10

"Substituted Alkenyl:" refers to an alkenyl group wherein one or more hydrogen atoms are each independently replaced with other substituents.

15 "Alkynyl:" refers to an unsaturated branched, straight chain or cyclic hydrocarbon group having at least one carbon-carbon triple bond. Typical alkynyl groups include, but are not limited to, ethynyl, propynyl, butynyl, isobutynyl, pentynyl, hexynyl and the like. In preferred embodiments, the alkynyl group is (C<sub>1</sub>-C<sub>6</sub>) alkynyl, with (C<sub>1</sub>-C<sub>3</sub>) being particularly preferred.

20

"Substituted Alkynyl:" refers to an alkynyl group wherein one or more hydrogen atoms are each independently replaced with other substituents.

25

"Alkoxy:" refers to an -OR group, where R is alkyl, alkenyl or alkynyl, as defined above.

30 "Aromatic moiety:" refers to a moiety having an unsaturated cyclic hydrocarbon group which has a conjugated (4n+2)  $\pi$  electron system. Typical aromatic moieties include, but are not limited to, benzene, naphthalene, anthracene, azulene, indacene, and the like. In preferred embodiments, the aromatic moiety contains 5-20 carbons in the ring system, with 5-10 carbon atoms being particularly preferred.

35

"Substituted Aromatic Moiety:" refers to an aromatic moiety wherein one or more hydrogen atoms are each independently replaced with other substituents.

5        "Heteroaromatic moiety:" refers to an aromatic moiety wherein one or more of the ring carbon atoms is replaced with another atom such as N, O or S. Typical heteroaromatic moieties include, but are not limited to, pyran, pyrazole, pyridine, pyrrole, pyrazine, pyridazine, pyrimidine,  
10 pyrrolizine, quinazoline, quinoline, quinolizine, quinoxaline, selenophene, thiophene, tellurophene, xanthene and the like.

      "Substituted Heteroaromatic moiety:" refers to a  
15 heteroaromatic moiety wherein one or more hydrogen atoms are each independently replaced with other substituents.

#### 5.1. THE TNF-R SUPERFAMILY

20        TNF exerts its biological activities by binding to two TNF-R: p55 and p75. A comparison of these receptors with several other cell surface receptors revealed certain shared structural features that led to their classification as a superfamily (Beutler et al., 1994, Science 264:667). The  
25 TNF-R superfamily members possess characteristic extracellular Cys-rich domains, yet share only about 25% sequence homology. There are at least ten members in this superfamily, including: TNF-R p55 and p75, TNF-R related protein (rp), CD40, Fas antigen (CD95), low-affinity nerve  
30 growth factor receptor (p75), CD27, CD30, 4-1BB and OX40 (Beutler et al., 1994, Ann. NY Acad. Sci. pp. 118-133; Gruss and Dower, 1995, Cytokines and Mol. Ther. 1:75-105).

      Loops and turns in many proteins have been shown to play functionally important roles in protein-protein interactions.  
35 In a specific embodiment illustrated by way of examples in Section 6, *infra*, cyclic peptides were designed from three

binding loops of TNF-R p55 which inhibited the binding of TNF to its cellular receptors. In particular, peptides designed from loop 1 of domain 3 exhibited the strongest inhibitory activities. When a peptide designed from this binding loop  
5 was used in combination with peptides designed from two other loops, no further increase in inhibitory effects were observed, indicating that loop 1 of domain 3 is a dominant ligand binding site in TNF-R.

Based on this finding, corresponding regions of other  
10 TNF-R superfamily members from which inhibitory peptides and peptide analogues can be designed are readily identified by amino acid sequence alignment with the three specific binding sites of TNF-R p55 (Figure 1). Since the dominant binding site of TNF-R p55 falls within amino acid residues #119 to  
15 136, which sequence starts and ends with Cys, the same region in each TNF-R superfamily member may be used to design peptides and peptide analogues that are within the scope of the present invention. In cases where the regions do not start or end with Cys, the region may extend to the next Cys.  
20 For example, the corresponding region in Fas is deleted, and thus this region in Fas starts at residue #97 and ends with #143. In the case of NGF-R, the region ends at the Cys at position at 135. Additionally, residues 74-81 and 97-110 may also be used to design additional peptides and peptide  
25 analogues within the scope of the present invention. Such compounds are then cyclized and modified at their termini with hydrophobic moieties as described in greater detail in the sections below.

30       **5.2. PEPTIDES AND PEPTIDE ANALOGUES DESIGNED FROM  
          BINDING LOOPS OF A TNF-R SUPERFAMILY MEMBER**

Generally, a compound of the present invention is a cyclic peptide or peptide analogue. The peptide or peptide analogue is modified at its termini with hydrophobic  
35 moieties. In embodiments wherein the compound is a peptide, the peptide corresponds in primary sequence to a binding loop of a member of the TNF-R superfamily or a portion thereof.

In a preferred embodiment, the peptide corresponds in primary sequence to a binding loop of TNF-R p55 or a portion thereof. In certain embodiments, one or more amino acid residues within the peptide are substituted with other amino acid residues. Typically, such amino acid substitutions are conservative, i.e., the amino acid residues are replaced with other amino acid residues having physical and/or chemical properties similar to the residues they are replacing. Preferably, conservative amino acid substitutions are those wherein an amino acid is replaced with another amino acid encompassed within the same designated class, as will be described more thoroughly below. In embodiments wherein the compound is a peptide analogue, the analogue is obtained by replacing at least one amide linkage in the peptide with a substituted amide or an isostere of amide.

In an illustrative embodiment, a compound of the invention has the following formula:



wherein:

AC is a peptide of 3-18 amino acid residues, preferably 5-8 amino acid residues, which corresponds in primary sequence to a binding loop of a TNF-R and which may optionally contain conservative amino acid substitutions, or an analogue thereof wherein at least one amide linkage is replaced with a substituted amide or an isostere of amide;

AB<sub>1</sub> is a moiety having a first functional group capable of forming a covalent linkage with one terminus of AC, a second functional group capable of forming a covalent linkage with AB<sub>2</sub>, and a third functional group capable of forming a covalent linkage with AA<sub>1</sub>;



AB<sub>2</sub> is a moiety having a first functional group capable of forming a covalent linkage with the second terminus of AC, a second functional group capable of forming a covalent linkage with AB<sub>1</sub> and a third functional group capable of forming a covalent linkage with AA<sub>2</sub>;

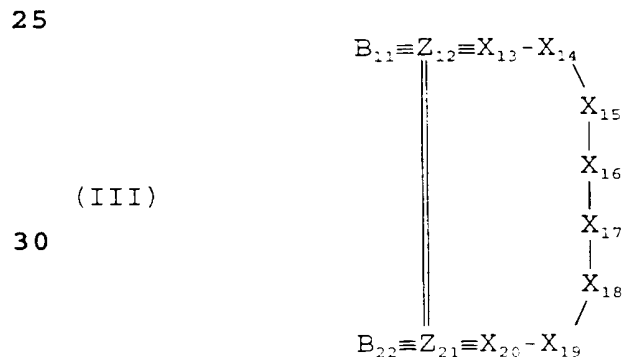
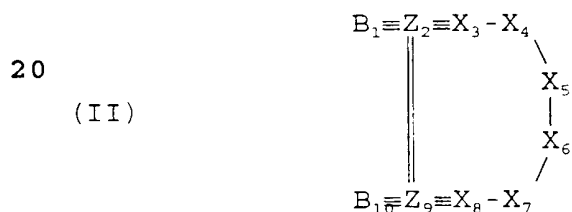
AA<sub>1</sub> is a moiety having hydrophobic properties and a functional group capable of forming a covalent linkage with the third functional group of AB<sub>1</sub>;

AA<sub>2</sub> is a moiety having a hydrophobic properties and a functional group capable of forming a covalent linkage with the third functional group of AB<sub>2</sub>;

"=" is a covalent linkage; and

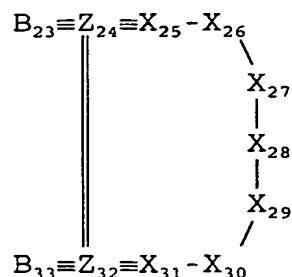
"≡" is a covalent linkage.

More specifically, the compounds of the invention are illustrated by three specific embodiments having the following formulae:



(IV)

5



The designation  $X_n$  in each case represents an amino acid at the specified position in the compound. Similarly, the designation  $Z_n$  represents an amino acid or other moiety which is capable of forming covalent linkages with other  $Z_n$ , such as disulfide bridges.

The amino acid residues denoted by  $X_n$  or  $Z_n$  may be the genetically encoded L-amino acids, naturally occurring non-genetically encoded L-amino acids, synthetic L-amino acids or D-enantiomers of all of the above. The amino acid notations used herein for the twenty genetically encoded L-amino acids and common non-encoded amino acids are conventional and are as follows:

20

25

30

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Amino Acid	One-Letter Symbol	Common Abbreviation
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro

	Amino Acid	One-Letter Symbol	Common Abbreviation
	Serine	S	Ser
	Threonine	T	Thr
5	Tryptophan	W	Trp
	Tyrosine	Y	Tyr
	Valine	V	Val
	$\beta$ -alanine		bAla
10	2,3-diaminopropionic acid		Dpr
	$\alpha$ -aminoisobutyric acid		Aib
	N-methylglycine (sarcosine)		MeGly
	Ornithine		Orn
	Citrulline		Cit
15	t-butylalanine		t-BuA
	t-butylglycine		t-BuG
	N-methylisoleucine		MeIle
	phenylglycine		Phg
	cyclohexylalanine		Cha
20	Norleucine		Nle
	naphthylalanine		Nal
	Pyridylalanine		
	3-benzothieryl alanine		
	4-chlorophenylalanine		Phe (4-Cl)
25	2-fluorophenylalanine		Phe (2-F)
	3-fluorophenylalanine		Phe (3-F)
	4-fluorophenylalanine		Phe (4-F)
	Penicillamine		Pen
30	1,2,3,4-tetrahydro- isoquinoline-3- carboxylic acid		Tic
	$\beta$ -2-thienylalanine		Thi
	Methionine sulfoxide		MSO
	Homoarginine		hArg
35	N-acetyl lysine		AcLys
	2,4-diamino butyric acid		Dbu
	p-aminophenylalanine		Phe (pNH <sub>2</sub> )

Amino Acid	One-Letter Symbol	Common Abbreviation
N-methylvaline		MeVal
Homocysteine		hCys
Homoserine		hSer
$\epsilon$ -amino hexanoic acid		Aha
$\delta$ -amino valeric acid		Ava
2,3-diaminobutyric acid		Dab

10 The compounds that are encompassed within the scope of  
 the invention are partially defined in terms of amino acid  
 residues of designated classes. The amino acids may be  
 generally categorized into three main classes: hydrophilic  
 amino acids, hydrophobic amino acids and Cysteine-like amino  
 15 acids, depending primarily on the characteristics of the  
 amino acid side chain. These main classes may be further  
 divided into subclasses. Hydrophilic amino acids include  
 amino acids having acidic, basic or polar side chains and  
 hydrophobic amino acids include amino acids having aromatic  
 20 or apolar side chains. Apolar amino acids may be further  
 subdivided to include, among others, aliphatic amino acids.  
 The definitions of the classes of amino acids as used herein  
 are as follows:

25 "Hydrophobic Amino Acid" refers to an amino acid having  
 a side chain that is uncharged at physiological pH and that  
 is repelled by aqueous solution. Examples of genetically  
 encoded hydrophobic amino acids include Ile, Leu and Val.  
 Examples of non-genetically encoded hydrophobic amino acids  
 30 include t-BuA.

"Aromatic Amino Acid" refers to a hydrophobic amino acid  
 having a side chain containing at least one ring having a  
 conjugated  $\pi$ -electron system (aromatic group). The aromatic  
 35 group may be further substituted with substituent groups such  
 as alkyl, alkenyl, alkynyl, hydroxyl, sulfanyl, nitro and  
 amino groups, as well as others. Examples of genetically

encoded aromatic amino acids include phenylalanine, tyrosine and tryptophan. Commonly encountered non-genetically encoded aromatic amino acids include phenylglycine, 2-naphthylalanine,  $\beta$ -2-thienylalanine, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, 4-chloro-phenylalanine, 2-fluorophenylalanine, 3-fluorophenylalanine and 4-fluorophenylalanine.

"Apolar Amino Acid" refers to a hydrophobic amino acid having a side chain that is generally uncharged at physiological pH and that is not polar. Examples of genetically encoded apolar amino acids include glycine, proline and methionine. Examples of non-encoded apolar amino acids include Cha.

15

"Aliphatic Amino Acid" refers to an apolar amino acid having a saturated or unsaturated straight chain, branched or cyclic hydrocarbon side chain. Examples of genetically encoded aliphatic amino acids include Ala, Leu, Val and Ile. Examples of non-encoded aliphatic amino acids include Nle.

20

"Hydrophilic Amino Acid" refers to an amino acid having a side chain that is attracted by aqueous solution. Examples of genetically encoded hydrophilic amino acids include Ser and Lys. Examples of non-encoded hydrophilic amino acids include Cit and hCys.

25

"Acidic Amino Acid" refers to a hydrophilic amino acid having a side chain pK value of less than 7. Acidic amino acids typically have negatively charged side chains at physiological pH due to loss of a hydrogen ion. Examples of genetically encoded acidic amino acids include aspartic acid (aspartate) and glutamic acid (glutamate).

30

"Basic Amino Acid" refers to a hydrophilic amino acid having a side chain pK value of greater than 7. Basic amino acids typically have positively charged side chains at

35

physiological pH due to association with hydronium ion. Examples of genetically encoded basic amino acids include arginine, lysine and histidine. Examples of non-genetically encoded basic amino acids include the non-cyclic amino acids  
5 ornithine, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid and homoarginine.

"Polar Amino Acid" refers to a hydrophilic amino acid having a side chain that is uncharged at physiological pH,  
10 but which has a bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Examples of genetically encoded polar amino acids include asparagine and glutamine. Examples of non-genetically encoded polar amino acids include citrulline, N-acetyl lysine  
15 and methionine sulfoxide.

"Cysteine-Like Amino Acid" refers to an amino acid having a side chain capable of forming a covalent linkage with a side chain of another amino acid residue, such as a  
20 disulfide linkage. Typically, cysteine-like amino acids generally have a side chain containing at least one thiol (SH) group. Examples of genetically encoded cysteine-like amino acids include cysteine. Examples of non-genetically encoded cysteine-like amino acids include homocysteine and  
25 penicillamine.

As will be appreciated by those having skill in the art, the above classification are not absolute -- several amino acids exhibit more than one characteristic property, and can  
30 therefore be included in more than one category. For example, tyrosine has both an aromatic ring and a polar hydroxyl group. Thus, tyrosine has dual properties and can be included in both the aromatic and polar categories. Similarly, in addition to being able to form disulfide  
35 linkages, cysteine also has apolar character. Thus, while not strictly classified as a hydrophobic or apolar amino

acid, in many instances cysteine can be used to confer hydrophobicity to a peptide.

Certain commonly encountered amino acids which are not genetically encoded of which the peptides and peptide analogues of the invention may be composed include, but are not limited to,  $\beta$ -alanine (b-Ala) and other omega-amino acids such as 3-aminopropionic acid (Dap), 2,3-diaminopropionic acid (Dpr), 4-aminobutyric acid and so forth;  $\alpha$ -aminoisobutyric acid (Aib);  $\epsilon$ -aminohexanoic acid (Aha);  $\delta$ -aminovaleric acid (Ava); N-methylglycine or sarcosine (MeGly); ornithine (Orn); citrulline (Cit); t-butylalanine (t-BuA); t-butylglycine (t-BuG); N-methylisoleucine (MeIle); phenylglycine (Phg); cyclohexylalanine (Cha); norleucine (Nle); 2-naphthylalanine (2-Nal); 4-chlorophenylalanine (Phe(4-Cl)); 2-fluorophenylalanine (Phe(2-F)); 3-fluorophenylalanine (Phe(3-F)); 4-fluorophenylalanine (Phe(4-F)); penicillamine (Pen); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic);  $\beta$ -2-thienylalanine (Thi); methionine sulfoxide (MSO); homoarginine (hArg); N-acetyl lysine (AcLys); 2,3-diaminobutyric acid (Dab); 2,3-diaminobutyric acid (Dbu); p-aminophenylalanine (Phe(pNH<sub>2</sub>)); N-methyl valine (MeVal); homocysteine (hCys) and homoserine (hSer). These amino acids also fall conveniently into the categories defined above.

The classifications of the above-described genetically encoded and non-encoded amino acids are summarized in Table 1, below. It is to be understood that Table 1 is for illustrative purposes only and does not purport to be an exhaustive list of amino acid residues which may comprise the peptides and peptide analogues described herein. Other amino acid residues which are useful for making the peptides and peptide analogues described herein can be found, e.g., in Fasman, 1989, CRC Practical Handbook of Biochemistry and Molecular Biology, CRC Press, Inc., and the references cited therein. Amino acids not specifically mentioned herein can be conveniently classified into the above-described categories on the basis of known behavior and/or their

characteristic chemical and/or physical properties as compared with amino acids specifically identified.

Table 1

5	<u>Table 1</u>		
	Classification	Genetically Encoded	Genetically Non-Encoded
	Hydrophobic		
	Aromatic	F, Y, W	Phg, Nal, Thi, Tic, Phe(4-Cl), Phe(2-F), Phe(3-F), Phe(4-F), Pyridyl Ala, Benzothienyl Ala
10	Apolar	M, G, P	
	Aliphatic	A, V, L, I	t-BuA, t-BuG, MeIle, Nle, MeVal, Cha, bAla, MeGly, Aib
	Hydrophilic		
	Acidic	D, E	
15	Basic	H, K, R	Dpr, Orn, hArg, Phe(p-NH <sub>2</sub> ), DBU, A <sub>2</sub> BU
	Polar	Q, N, S, T, Y	Cit, AcLys, MSO, hSer
	Cysteine-Like	C	Pen, hCys, $\beta$ -methyl Cys

The designation Z<sub>n</sub> in each case represents an amino acid or other moiety capable of forming covalent linkages with other Z<sub>n</sub> so as to allow cyclization of the peptide. Examples of amino acid residues which are capable of forming covalent linkages with one another include cysteine-like amino acids such Cys, hCys,  $\beta$ -methyl Cys and Pen, which are capable of forming disulfide bridges with one another. Preferred cysteine-like amino acid residues include Cys and Pen.

Amino acids used to cyclize a peptide need not be cysteine-like amino acids. Pairs of amino acids that have side chain functional groups capable of forming covalent linkages with one another can also be used. Such pairs of functional groups are known to those of skill in the art and include, *inter alia*, -COOH and -OH, -COOH and -NH<sub>2</sub>, and -COOH and -SH. Thus, pairs of amino acids that can be used to cyclize a peptide include, *inter alia*, Asp and Lys; Glu and Lys; Asp and Arg; Glu and Arg; Asp and Ser; Glu and Ser; Asp and Thr; Glu and Thr; Asp and Cys; and Glu and Cys. Other



pairs of amino acids which can be used to cyclize the peptide will be apparent to those skilled in the art.

It will also be recognized that  $Z_n$  groups used to cyclize a peptide need not be amino acids. Thus,  $Z_n$  may be any molecule having three functional groups -- one functional group capable of forming a covalent linkage with a terminus of the peptide, a second functional group capable of forming a covalent linkage with the second functional group of another  $Z_n$ , and a third functional group capable of forming a covalent linkage with hydrophobic moieties  $B_n$ . Molecules having suitable functional groups will be apparent to those skilled in the art. Examples of functional groups capable of forming a covalent linkage with the amino terminus of a peptide include carboxylic acids and esters. Examples of functional groups capable of forming a covalent linkage with the carboxyl terminus of a peptide include -OH, -SH, -NH<sub>2</sub> and -NHR where R is (C<sub>1</sub>-C<sub>6</sub>) alkyl, (C<sub>1</sub>-C<sub>6</sub>) alkenyl and (C<sub>1</sub>-C<sub>6</sub>) alkynyl.

A variety of interlinkages useful to cyclize a peptide can be generated by reaction between two  $Z_n$ .  $Z_n$  with functional groups suitable for forming such interlinkages, as well as reaction conditions suitable for forming such interlinkages, will be apparent to those of skill in the art. Preferably, the reaction conditions used to cyclize the peptides are sufficiently mild so as not to degrade or otherwise damage the peptide. Suitable groups for protecting the various functionalities as necessary are well known in the art (see, e.g., Greene & Wuts, 1991, 2nd ed., John Wiley & Sons, NY), as are various reaction schemes for preparing such protected molecules.

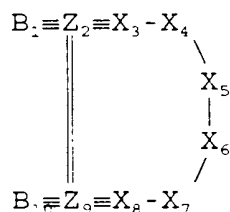
The designation  $B_n$  in each case represents a hydrophobic moiety. While not intending to be bound by any particular theory, it is believed that when placed in aqueous solution, these hydrophobic moieties interact so as to confer the peptide with structural stability. A significant hydrophobic interaction for conferring structural stability is thought to be stacking of aromatic rings. Thus, in a preferred

embodiment, each  $B_n$  designates a peptide of 1-6 amino acids, at least one of which is an aromatic amino acid or an aromatic or heteroaromatic moiety.  $B_n$  may be illustrated as  $X_{32}-X_{33}-X_{34}-X_{35}-X_{36}-X_{37}$  wherein  $X_n$  is an amino acid, at least one of which is an aromatic amino acid. Preferably,  $X_{32}-X_{33}-X_{34}$  are absent and  $X_{37}$  is an aromatic amino acid. More preferably,  $X_{32}-X_{33}-X_{34}-X_{35}-X_{36}$  are absent and  $X_{37}$  is an aromatic amino acid. Suitable aromatic amino acids include Tyr, Phe and Trp, with Tyr and Phe being preferred. Suitable aromatic or heteroaromatic moieties include phenyl, naphthyl, purine, pyrimidine, and the like.

In the peptides of formulae (II) - (IV), the symbol "-" between amino acid residues  $X_n$  generally designates a backbone interlinkage. Thus, the symbol "-" usually designates an amide linkage ( $-C(O)-NH$ ). It is to be understood, however, that in all of the peptides described in the specific embodiments herein, one or more amide linkages may optionally be replaced with a linkage other than amide, preferably a substituted amide or an isostere of an amide linkage. Thus, while the various  $X_n$  have generally been described in terms of amino acids, one having skill in the art will recognize that in embodiments having non-amide linkages, the term "amino acid" refers to other bifunctional moieties having side-chain groups similar to the side chains of the amino acids. For example, in embodiments having non-amide linkages, the phrase "acidic amino acid" refers to a bifunctional molecule capable of forming the desired backbone interlinkages and which has a side chain group similar to the side chain of an acidic amino acid. Substituted amides generally include groups of the formula  $-C(O)-NR$ , where R is  $(C_1-C_6)$  alkyl,  $(C_1-C_6)$  alkenyl,  $(C_1-C_6)$  alkynyl, substituted  $(C_1-C_6)$  alkyl, substituted  $(C_1-C_6)$  alkenyl or substituted  $(C_1-C_6)$  alkynyl. Isosteres of amide generally include, but are not limited to,  $-CH_2NH-$ ,  $-CH_2S-$ ,  $-CH_2CH_2-$ ,  $-CH=CH-$  (cis and trans),  $-C(O)CH_2-$ ,  $-CH(OH)CH_2-$  and  $-CH_2SO-$ .

Compounds having such linkages and methods for preparing such compounds are well-known in the art (see, e.g., Spatola,

- 30 (II)



wherein:

B<sub>1</sub> and B<sub>10</sub> are each independently a peptide of 1-6 amino acids, at least one of which is a hydrophobic amino acid, an aromatic moiety or a heteroaromatic moiety;

5 Z<sub>2</sub> is a moiety that is capable of forming a covalent linkage with B<sub>1</sub>, X<sub>3</sub> and Z<sub>9</sub>;

Z<sub>9</sub> is a moiety that is capable of forming a covalent linkage with B<sub>10</sub>, X<sub>8</sub> and Z<sub>2</sub>;

X<sub>3</sub> is absent or a hydrophilic amino acid;

X<sub>4</sub> is a hydrophobic amino acid;

10 X<sub>5</sub> is a hydrophilic amino acid;

X<sub>6</sub> is a hydrophilic amino acid;

X<sub>7</sub> is a hydrophobic or hydrophilic amino acid;

X<sub>8</sub> is a hydrophobic or hydrophilic amino acid;

15 "-" is an amide, substituted amide or an isostere of amide thereof;

"=" is a covalent linkage; and

"≡" is a covalent linkage.

In a preferred embodiment of the invention, the compounds are those of formula (II) wherein:

20 B<sub>1</sub> and B<sub>10</sub> are each independently a peptide of 1-3 amino acids, at least one of which is an aromatic amino acid;

Z<sub>2</sub> and Z<sub>9</sub> are each independently a Cys-like amino acid;

X<sub>3</sub> is absent or an acidic amino acid;

X<sub>4</sub> is an aromatic or apolar amino acid;

25 X<sub>5</sub> is a polar amino acid;

X<sub>6</sub> is a polar amino acid;

X<sub>7</sub> is an aromatic or polar amino acid;

X<sub>8</sub> is an aromatic, apolar or polar amino acid;

"-" is an amide linkage;

30 "=" is a disulfide linkage; and

"≡" is an amide linkage.

In a particularly preferred embodiment, the compounds of the invention are those of formula (II) wherein:

B<sub>1</sub> and B<sub>10</sub> are each independently Tyr or Phe;

35 Z<sub>2</sub> and Z<sub>9</sub> are each Cys;

X<sub>3</sub> is absent or Glu;

X<sub>4</sub> is Trp or Leu;

$X_5$  is Ser;

$X_6$  is Gln;

$X_7$  is Tyr or Asn;

$X_8$  is Tyr or Leu;

5     "-" is an amide linkage;

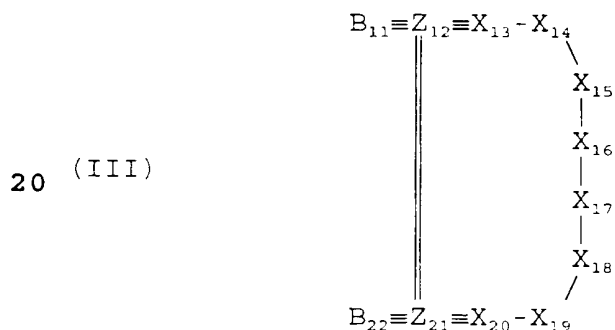
"=" is a disulfide linkage; and

"≡" is an amide linkage.

Particularly preferred peptides of the invention include the following:

10	YCELSQYLCY	(SEQ ID NO:12)
	YC WSQNLCY	(SEQ ID NO:13)
	YC WSQNYCY	(SEQ ID NO:14)
	YC WSQYLCY	(SEQ ID NO:15)

15     In a second illustrative embodiment, the compounds of formula (III) are defined as follows:



25     wherein:

$B_{11}$  and  $B_{22}$  are each independently a peptide of 1-6 amino acids, at least one of which is a hydrophobic amino acid, an aromatic moiety or a heteroaromatic moiety;

30      $Z_{12}$  is a moiety that is capable of forming a covalent linkage with  $B_{11}$ ,  $X_{13}$  and  $Z_{21}$ ;

$Z_{21}$  is a moiety that is capable of forming a covalent linkage with  $B_{22}$ ,  $X_{20}$  and  $Z_{12}$ ;

$X_{13}$  is absent or hydrophobic amino acid;

$X_{14}$  is absent or a hydrophilic amino acid;

35      $X_{15}$  is a hydrophilic or hydrophobic amino acid;

$X_{16}$  is a hydrophilic amino acid;

$X_{17}$  is absent or a hydrophobic amino acid;

X<sub>18</sub> is a hydrophilic amino acid;  
X<sub>19</sub> is a hydrophilic amino acid;  
X<sub>20</sub> is a hydrophilic amino acid;  
"-" is an amide, a substituted amide or an isostere of  
5 amide thereof;  
"=" is a covalent linkage; and  
"≡" is a covalent linkage.

In a preferred embodiment, the compounds are those of  
formula (III) wherein:

10 B<sub>11</sub> and B<sub>22</sub> are each independently a peptide of 1-3 amino  
acids, at least one of which is an aromatic amino acid;  
Z<sub>12</sub> and Z<sub>21</sub> are each independently a Cys-like amino acid;  
X<sub>13</sub> is absent or an aromatic amino acid;  
X<sub>14</sub> is absent or a polar amino acid;  
15 X<sub>15</sub> is a basic, polar or apolar amino acid;  
X<sub>16</sub> is a polar amino acid;  
X<sub>17</sub> is absent or an apolar amino acid ;  
X<sub>18</sub> is an acidic amino acid;  
X<sub>19</sub> is a polar amino acid;  
20 X<sub>20</sub> is a basic amino acid;  
"-" is an amide linkage;  
"=" is a disulfide linkage; and  
"≡" is an amide linkage.

In a particularly preferred embodiment, the compounds  
25 are those of formula (III), wherein:

B<sub>11</sub> and B<sub>22</sub> are each independently Tyr or Phe;  
Z<sub>12</sub> and Z<sub>21</sub> are each Cys;  
X<sub>13</sub> is absent or Phe;  
X<sub>14</sub> is absent or Thr;  
30 X<sub>15</sub> is Ala, Asn or Arg;  
X<sub>16</sub> is Ser;  
X<sub>17</sub> is absent or Val;  
X<sub>18</sub> is Glu;  
X<sub>19</sub> is Asn;  
35 X<sub>20</sub> is Arg or His;  
"-" is an amide linkage;  
"=" is a disulfide linkage; and

"=" is an amide linkage.

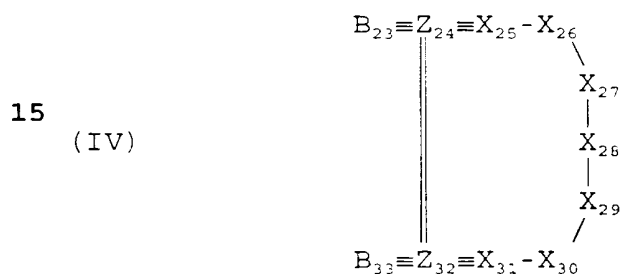
Particularly preferred peptides of the invention include the following:

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5      YC FTASENH CY          (SEQ. ID NO:16)
      YC FTNSENH CY          (SEQ. ID NO:17)
      YC FTRSENH CY          (SEQ. ID NO:18)
      FC      ASENH CY          (SEQ. ID NO:19)
      YC      ASENH CY          (SEQ. ID NO:20)
      FC      NSENH CY          (SEQ. ID NO:21)
      FC      NSENR CY          (SEQ. ID NO:22)
      FC      NSVENR CY          (SEQ. ID NO:23)

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10 In a third illustrative embodiment, the compounds of  
formula (IV) are defined as follows:



20

wherein:

B<sub>23</sub> and B<sub>33</sub> are each independently a peptide of 1-6 amino acids, at least one of which is a hydrophobic amino acid, an aromatic moiety or a heteroaromatic moiety;

$Z_{24}$  is a moiety that is capable of forming a covalent linkage with  $B_{23}$ ,  $X_{25}$  and  $Z_{32}$ ;

$Z_{32}$  is a moiety that is capable of forming a covalent linkage with  $B_{33}$ ,  $X_{31}$  and  $Z_{24}$ ;

30 X<sub>25</sub> is absent or a hydrophilic amino acid;

X<sub>26</sub> is a hydrophilic amino acid;

X<sub>27</sub> is a hydrophilic amino acid;

X<sub>20</sub> is a hydrophobic amino acid;

X<sub>79</sub> is a hydrophobic amino acid;

35 X<sub>36</sub> is absent or a hydrophilic amino acid;

X<sub>3</sub> is absent or a hydrophobic amino acid;

"-" is an amide, a substituted amide or an isostere of amide;

"=" is a covalent linkage; and

"≡" is a covalent linkage.

- 5 In a preferred embodiment, the compounds are those of formula (IV) wherein:

B<sub>23</sub> and B<sub>33</sub> are each independently a peptide of 1-3 amino acids, at least one of which is an aromatic amino acid;

Z<sub>24</sub> and Z<sub>32</sub> are each independently a Cys-like amino acid;

- 10 X<sub>25</sub> is absent or a basic amino acid;

X<sub>26</sub> is a basic amino acid;

X<sub>27</sub> is an acidic amino acid;

X<sub>28</sub> is an apolar amino acid;

X<sub>29</sub> is an apolar amino acid;

- 15 X<sub>30</sub> is absent or a polar amino acid;

X<sub>31</sub> is absent or an apolar amino acid;

"-" is an amide linkage;

"=" is a disulfide linkage; and

"≡" is an amide linkage.

- 20 In a particularly preferred embodiment, the compounds of the invention or analogues thereof are those of formula (IV), wherein:

B<sub>23</sub> and B<sub>33</sub> are each independently Tyr or Phe;

Z<sub>24</sub> and Z<sub>32</sub> are each Cys;

- 25 X<sub>25</sub> is absent or Arg;

X<sub>26</sub> is Lys;

X<sub>27</sub> is Glu;

X<sub>28</sub> is Leu, Pro or Met;

X<sub>29</sub> is Gly;

- 30 X<sub>30</sub> is absent or Gln;

X<sub>31</sub> is absent or Val;

"-" is an amide linkage;

"=" is a disulfide linkage; and

"≡" is an amide linkage.

- 35 Particularly preferred peptides of the invention include the following:

YC RKELGQV CY

(SEQ. ID NO:24)



YC KEPGQ CY (SEQ. ID NO:25)  
YC RKEMG CY (SEQ. ID NO:26)  
FC RKEMG CY (SEQ. ID NO:27)

In all of the aforementioned embodiments of the invention, it is to be understood that the phrase "amino acid" also refers to bifunctional moieties having amino acid-like side chains, as previously described.

Generally, active peptides or peptide analogues of the invention are those that exhibit at least about 15% inhibition of TNF-R:TNF interactions as measured in vitro assays such as those described in Section 6, *infra*. Preferably, active peptides of the invention or analogues thereof will exhibit at least about 20% to 50% or even 80% or more inhibition of TNF-R:TNF- $\alpha$  binding interactions.

15

### 5.3. PREPARATION OF PEPTIDES AND PEPTIDE ANALOGUES

#### 5.3.1. CHEMICAL SYNTHESIS

The peptides of the invention or analogues thereof, may be prepared using virtually any art-known technique for the preparation of peptides and peptide analogues. For example, the peptides may be prepared in linear or non-cyclized form using conventional solution or solid phase peptide syntheses and cyclized using standard chemistries. Preferably, the chemistry used to cyclize the peptide will be sufficiently mild so as to avoid substantially degrading the peptide. Suitable procedures for synthesizing the peptides described herein as well as suitable chemistries for cyclizing the peptides are well known in the art.

Formation of disulfide linkages, if desired, is generally conducted in the presence of mild oxidizing agents. Chemical, enzymatic or photolytic oxidation agents may be used. Various methods are known in the art, including those described, for example, by Tam, J.P. et al., 1979, Synthesis 955-957; Stewart et al., 1984, Solid Phase Peptide Synthesis, 2d Ed., Pierce Chemical Company Rockford, IL; Ahmed et al., 1975, J. Biol. Chem. 250:8477-8482; and Pennington et al., 1991 Peptides 1990 164-166, Giralt and Andreu, Eds., ESCOM

Leiden, The Netherlands. An additional alternative is described by Kamber et al., 1980, Helv Chim Acta 63:899-915. A method conducted on solid supports is described by Albericio, 1985, Int. J. Peptide Protein Res. 26:92-97. Any  
5 of these methods may be used to form disulfide linkages in the peptides of the invention. Preferred methods for effecting disulfide-bridge formation for the peptides described herein are provided in the examples.

#### 10 5.3.2. RECOMBINANT SYNTHESIS

If the peptide is composed entirely of gene-encoded amino acids, or a portion of it is so composed, the peptide or the relevant portion may also be synthesized using conventional recombinant genetic engineering techniques. The  
15 isolated peptides, or segments thereof, are then condensed, and oxidized, as previously described, to yield a cyclic peptide.

For recombinant production, a polynucleotide sequence encoding a linear form of the peptide is inserted into an  
20 appropriate expression vehicle, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence, or in the case of an RNA viral vector, the necessary elements for replication and translation. The expression vehicle is then transfected into  
25 a suitable target cell which will express the linear form of the cyclic peptide. Depending on the expression system used, the expressed peptide is then isolated by procedures well-established in the art. Methods for recombinant protein and peptide production are well known in the art (see, e.g.,  
30 Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.).

A variety of host-expression vector systems may be  
35 utilized to express the peptides described herein. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage DNA or

plasmid DNA expression vectors containing an appropriate coding sequence; yeast or filamentous fungi transformed with recombinant yeast or fungi expression vectors containing an appropriate coding sequence; insect cell systems infected  
5 with recombinant virus expression vectors (e.g., baculovirus) containing an appropriate coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus or tobacco mosaic virus) or transformed with recombinant plasmid expression vectors  
10 (e.g., Ti plasmid) containing an appropriate coding sequence; or animal cell systems.

The expression elements of the expression systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable  
15 transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage  $\lambda$ , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may  
20 be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedron promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for  
25 the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses  
30 (e.g., the adenovirus late promoter; the vaccinia virus 7.5 K promoter) may be used; when generating cell lines that contain multiple copies of expression product, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

35 In cases where plant expression vectors are used, the expression of sequences encoding the peptides of the invention may be driven by any of a number of promoters. For

example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant  
5 promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843) or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into  
10 plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, e.g., Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-  
15 463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

In one insect expression system that may be used to produce the peptides of the invention, *Autographa californica* nuclear polyhydrosis virus (AcNPV) is used as a vector to  
20 express the foreign genes. The virus grows in *Spodoptera frugiperda* cells. A coding sequence may be cloned into non-essential regions (for example the polyhedron gene) of the virus and placed under control of an AcNPV promoter (for example, the polyhedron promoter). Successful insertion of a  
25 coding sequence will result in inactivation of the polyhedron gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedron gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene  
30 is expressed. (e.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051). Further examples of this expression system may be found in Current Protocols in Molecular Biology, Vol. 2, Ausubel et al., eds., Greene Publish. Assoc. & Wiley Interscience.

35 In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, a coding sequence

may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination.

5 Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing peptide in infected hosts. (e.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Alternatively, the vaccinia 7.5 K  
10 promoter may be used, (see, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79:4927-4931).

Other expression systems for producing linear or non-  
15 cyclized forms of the cyclic peptides of the invention will be apparent to those having skill in the art.

#### 5.3.3. PURIFICATION OF THE PEPTIDES AND PEPTIDE ANALOGUES

The peptides and peptide analogues of the invention can  
20 be purified by art-known techniques such as high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, affinity chromatography and the like. The actual conditions used to purify a particular peptide or analogue will depend, in part, on factors such as net charge,  
25 hydrophobicity, hydrophilicity, etc., and will be apparent to those having skill in the art.

For affinity chromatography purification, any antibody which specifically binds the peptides or peptide analogues may be used. For the production of antibodies, various host  
30 animals, including but not limited to rabbits, mice, rats, etc., may be immunized by injection with a linear or cyclic peptide. The peptide may be attached to a suitable carrier, such as BSA, by means of a side chain functional group or linkers attached to a side chain functional group. Various  
35 adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as

aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacilli  
5 Calmette-Guerin) and *Corynebacterium parvum*.

Monoclonal antibodies to a peptide may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally  
10 described by Koehler and Milstein, 1975, Nature 256:495-497, the human B-cell hybridoma technique, Kosbor et al., 1983, Immunology Today 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030 and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy,  
15 Alan R. Liss, Inc., pp. 77-96 (1985)). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the  
20 genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be  
25 adapted to produce cyclic peptide-specific single chain antibodies.

Antibody fragments which contain deletions of specific binding sites may be generated by known techniques. For example, such fragments include but are not limited to F(ab')<sub>2</sub>  
30 fragments, which can be produced by pepsin digestion of the antibody molecule and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science 246:1275-1281) to allow rapid and  
35 easy identification of monoclonal Fab fragments with the desired specificity for the cyclic peptide of interest.

The antibody or antibody fragment specific for the desired cyclic peptide can be attached, for example, to agarose, and the antibody-agarose complex is used in immunochromatography to purify cyclic peptides of the invention. See, Scopes, 1984, Protein Purification: Principles and Practice, Springer-Verlag New York, Inc., NY, Livingstone, 1974, Methods Enzymology: Immunoaffinity Chromatography of Proteins 34:723-731.

10       **5.4. USES OF PEPTIDES AND PEPTIDE ANALOGUES  
          DESIGNED FROM A TNF-R SUPERFAMILY MEMBER**

The compounds of the present invention are useful for inhibiting the biological activities of ligands for members of the TNF-R superfamily. Ligands for all TNF-R superfamily members have been identified and isolated, and they are also classified into a superfamily based on their structural similarities. All members of this ligand family are capable of regulating diverse cellular responses, including proliferation, activation, differentiation, and even cell death by apoptosis or cytotoxicity. Since all TNF-R superfamily members are expressed on lymphocytes, particularly T cells, their corresponding ligands are able to regulate immune responses. A second commonly shared feature of all TNF ligand superfamily members is their ability to induce cell death.

As an illustration, CD40 is expressed on B cells and cross-linking of this receptor by its ligand induces B cell activation, including IgE secretion. Therefore, inhibition of CD40 interactions with its ligand down-regulates B cell responses, and may be used to treat autoimmunity and hypersensitivity. In contrast, the Fas ligand cross-links Fas antigen (CD95) expressed on activated T cells and B cells, and induces cell death. The inhibition of Fas ligand and Fas antigen interactions has been used to prevent transplantation rejections. Fas ligand has been proposed as a potential immunosuppressive agent, based on the observation that the expression of the Fas ligand by Sertoli cells

accounted for the immune-privileged nature of testis by inducing apoptosis of Fas-expressing T cells (Bellgrau et al., 1995, Nature 377:360). Therefore, compounds of the invention which inhibit the binding of Fas with its ligand  
5 may be used to augment immune responses in certain settings.

In a preferred embodiment of the invention, a compound of the invention inhibits TNF binding to its receptors. Since TNF- $\alpha$  and TNF- $\beta$  bind to the same receptors, such compounds are expected to inhibit both TNF- $\alpha$  and TNF- $\beta$   
10 activities. Such compounds may be used for the treatment of pathological conditions in which the biological activities of TNF play an active role. Such conditions, include, but are not limited to, acute and chronic inflammation, cachexia, septic shock, graft-versus-host disease, transplantation  
15 rejection, hypersensitivity, immune complex disease, malaria, and autoimmunity such as multiple sclerosis, rheumatoid arthritis, periodontal disease and non-insulin-dependent diabetes.

#### 20 5.4.1. FORMULATION AND ROUTE OF ADMINISTRATION

The compounds of the invention, may be administered to a subject *per se* or in the form of a pharmaceutical composition. Pharmaceutical compositions comprising the compounds of the invention may be manufactured by means of  
25 conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers, diluents, excipients or  
30 auxiliaries which facilitate processing of the active peptides or peptide analogues into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For topical administration the compounds of the  
35 invention may be formulated as solutions, gels, ointments, creams, suspensions, etc. as are well-known in the art.



Systemic formulations include those designed for administration by injection, e.g. subcutaneous, intravenous, intramuscular, intrathecal or intraperitoneal injection, as well as those designed for transdermal, transmucosal, oral or  
5 pulmonary administration.

For injection, the compounds of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. The  
10 solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Alternatively, the compounds may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

15 For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be readily formulated by combining the active peptides or peptide  
20 analogues with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. For  
25 oral solid formulations such as, for example, powders, capsules and tablets, suitable excipients include fillers such as sugars, such as lactose, sucrose, mannitol and sorbitol; cellulose preparations such as maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth,  
30 methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP); granulating agents; and binding agents. If desired, disintegrating agents may be added, such as the cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof  
35 such as sodium alginate.

If desired, solid dosage forms may be sugar-coated or enteric-coated using standard techniques.

For oral liquid preparations such as, for example, suspensions, elixirs and solutions, suitable carriers, excipients or diluents include water, glycols, oils, alcohols, etc. Additionally, flavoring agents, 5 preservatives, coloring agents and the like may be added.

For buccal administration, the compounds may take the form of tablets, lozenges, etc. formulated in conventional manner.

For administration by inhalation, the compounds for use 10 according to the present invention are conveniently delivered in the form of an aerosol spray from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable 15 gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as 20 lactose or starch.

The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, e.g, containing conventional suppository bases such as cocoa butter or other glycerides.

25 In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the 30 compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

35 Alternatively, other pharmaceutical delivery systems may be employed. Liposomes and emulsions are well known examples of delivery vehicles that may be used to deliver peptides and

peptide analogues of the invention. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, 5 such as semipermeable matrices of solid polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for 10 a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

As the compounds of the invention may contain charged 15 side chains or termini, they may be included in any of the above-described formulations as the free acids or bases or as pharmaceutically acceptable salts. Pharmaceutically acceptable salts are those salts which substantially retain the antimicrobial activity of the free bases and which are 20 prepared by reaction with inorganic acids. Pharmaceutical salts tend to be more soluble in aqueous and other protic solvents than are the corresponding free base forms.

#### 5.4.2. EFFECTIVE DOSAGES

25 The compounds of the invention will generally be used in an amount effective to achieve the intended purpose. For use to treat or prevent TNF-associated disorders, the compounds of the invention, or pharmaceutical compositions thereof, are administered or applied in a therapeutically effective 30 amount. By therapeutically effective amount is meant an amount effective ameliorate or prevent the symptoms, or prolong the survival of, the patient being treated. Determination of a therapeutically effective amount is well within the capabilities of those skilled in the art, 35 especially in light of the detailed disclosure provided herein.

For systemic administration, a therapeutically effective dose can be estimated initially from *in vitro* assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the  $IC_{50}$  as  
5 determined in cell culture (*i.e.*, the concentration of test compound that inhibits 50% of TNF-R:TNF-binding interactions). Such information can be used to more accurately determine useful doses in humans.

Initial dosages can also be estimated from *in vivo* data,  
10 *e.g.*, animal models, using techniques that are well known in the art. One having ordinary skill in the art could readily optimize administration to humans based on animal data.

Dosage amount and interval may be adjusted individually to provide plasma levels of the compounds which are  
15 sufficient to maintain therapeutic effect. Usual patient dosages for administration by injection range from about 0.1 to 5 mg/kg/day, preferably from about 0.5 to 1 mg/kg/day. Therapeutically effective serum levels may be achieved by administering multiple doses each day.

20 In cases of local administration or selective uptake, the effective local concentration of the compounds may not be related to plasma concentration. One having skill in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

25 The amount of compound administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

The therapy may be repeated intermittently while  
30 symptoms detectable or even when they are not detectable. The therapy may be provided alone or in combination with other drugs. In the case of TNF-associated disorders, the drugs that may be used in combination with the compounds of the invention include, but are not limited to, steroid and  
35 non-steroid anti-inflammatory agents.

### 5.4.3. TOXICITY

Preferably, a therapeutically effective dose of the compounds described herein will provide therapeutic benefit without causing substantial toxicity.

5 Toxicity of the compounds described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by determining the LD<sub>50</sub> (the dose lethal to 50% of the population) or the LD<sub>100</sub> (the dose lethal to 100% of the population). The dose ratio  
10 between toxic and therapeutic effect is the therapeutic index. Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a dosage range that is not toxic for use in human. The dosage of the  
15 compounds described herein lies preferably within a range of circulating concentrations that include the effective dose with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of  
20 administration and dosage can be chosen by the individual physician in view of the patient's condition. (See, e.g., Fingl et al., 1975, In: The Pharmacological Basis of Therapeutics, Ch.1, p.1).

25 The invention having been described, the following examples are offered by way of illustration and not limitation.

## 30 6. EXAMPLE: EXOCYCLIC PEPTIDES DERIVED FROM TNF-R ANTAGONIZE TNF- $\alpha$ ACTIVITIES

### 6.1. MATERIALS AND METHODS

#### 6.1.1. REAGENTS

Human recombinant TNF- $\alpha$  and the <sup>125</sup>I-labeled TNF- $\alpha$  were  
35 obtained from Amersham Life Science, Inc. (Arlington Heights, IL). TNF-R(I) or p55 extracellular domain-IgG heavy chain chimeric protein was prepared by expression of a cDNA

construct (Peppel et al., 1991, J. Exp. Med. 174:1483; Williams et al., 1995, Immunol. 84:433). Anti-TNF- $\alpha$  monoclonal antibody was made according to Doring et al. (1994, Molecular Immunol. 31:1059) and anti-TNF-R(I) monoclonal antibody (htr-9) was obtained from BMA Biomedicals AG (Augst, Switzerland).

#### 6.1.2. MOLECULAR MODELING

Computer modeling was performed using Quanta 4.0 (Molecular Simulation Inc., MA). The model peptides were constructed from their sequences and folded using CHARMM. The side chains of amino acid residues were first positioned to permitted conformation using Ponders rotamer (Ponder et al., 1987, J. Mol. Biol. 193:775-791) database provided in QUANTA. Then the folded peptides were minimized to convergence with dielectric constant set to 80.

The crystal structure of the TNF- $\beta$ /TNF-R(I) complex (Banner et al., 1993, Cell 73:431) was utilized to determine the binding sites of TNF-R for TNF- $\alpha$ . The first (56-73) and second (76-83) loops of domain 2 and the first loop (107-114) of domain 3 of the TNF-R were explored for use in designing peptides. The essential amino acid sequences of TNF-R for binding interactions with TNF- $\alpha$  were identified as structural templates by superimposing TNF- $\alpha$  to TNF $\beta$  complexed with its cognate receptor. Then, 5-8 amino acid-long peptides derived from TNF-R as shown in Table 2 were used as templates for the design of exocyclic peptides. Additional peptides were derived from CDR sequences of a light chain of an anti-TNF- $\alpha$  neutralizing antibody, CDR1L of Di62 (Doring et al., 1994, Mol. Immunol. 31:1059). Exocyclic modifications such as peptide cyclization and addition of aromatic amino acids such as Phe and Tyr to the ends of each peptide were performed as described (Zhang et al., 1996, Nature Biotech. 14:472; Zhang et al., 1997, Nature Biotech. 15:150).

35

### 6.1.3. PEPTIDE SYNTHESIS, CYCLIZATION AND PURIFICATION

Linear peptides were synthesized by solid-phase methods, deprotected, and released from the resin utilizing standard methodology. Peptides were precipitated and purified by high performance liquid chromatography (HPLC) utilizing a C18 column and then lyophilized. The purity of such peptides was greater than 95% as measured by HPLC analysis.

The peptides containing internal Cys residues were oxidized by dissolving them at 100 µg/ml in distilled water adjusted or buffered to pH 8.0 - 8.5, for example, by (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub> with stirring and exposure to air at 4°C for <10 days until 95% formation of intramolecular disulfide bonds had been confirmed by DTNB (Sigma, St. Louis, MO) which determined free sulfhydryls in peptides (Habeeb, 1973, Anal. Bioch. 56:60; Angeletti et al., 1996, In Techniques in Protein Chemistry VII, Ed. Marsak, Academic Press, San Diego, CA, pp. 81-91). Briefly, peptides (100 µg/ml, 50 µl) and DTNB (10mM, 50 µl) were added to 0.1 M sodium phosphate buffer (pH 8.0, 1 ml), incubated in the dark for 30 minutes, and the absorbance at 420 nm was determined and compared with the linear unoxidized peptides.

The cyclized peptides were lyophilized, purified by HPLC utilizing a C18 preparative column and a size exclusion column Protein-Pak 60 (Waters, Milford, MA). The purity of the peptides was shown to be greater than 95% by HPLC analysis. The concentration of each cyclized peptide was calculated based on UV intensity versus the corresponding linear peptide by HPLC analysis.

### 6.1.4. COMPETITIVE RADIORECEPTOR ASSAY

TNF-R-IgG chimeric protein (100 ng/ml) diluted in 100 µl phosphate buffered saline (PBS) was immobilized onto MicroTest III flexible assay plates (Becton Dickinson, San Jose, CA) by incubation at 4°C overnight. After blocking with PBS containing 1% bovine serum albumin (BSA) for 2 hr. at room temperature and subsequent washing with PBS containing 0.1% Tween 20 (PBS-Tw), <sup>125</sup>I-labeled-TNF-α(1ng) was

preincubated with a peptide solution (100  $\mu$ l) in PBS for 1 hr. at 37°C and was added to the TNF-R-coated wells. After 2 hr. incubation at 37°C, the plate was washed with PBS-Tw, and bound radioactivity was measured in Cobra gamma counter 5 (Packard Instruments, Meriden, CT).

#### 6.1.5. FLOW CYTOMETRY

A human leukemia cell line, U937, was maintained in RPMI-1640 medium supplemented with 10% FCS. In order to 10 quantitate the binding of TNF- $\alpha$  to its cognate receptor expressed on U937 cells,  $1 \times 10^5$  cells were re-suspended in PBS containing 0.5% BSA and 0.05% NaN<sub>3</sub> (binding buffer). TNF- $\alpha$  (2.5ng) was preincubated with a peptide solution (50  $\mu$ l) in PBS for 1 hr. at 37°C and was added to the cells for 1 hr. at 15 4°C at a final TNF- $\alpha$  concentration of 50 ng/ml. Then, 50  $\mu$ l of an anti-TNF receptor antibody at 3  $\mu$ g/ml in binding buffer were added to the cells for 1 hr. at 4°C. The cells were washed in binding buffer and stained with 50  $\mu$ l (10  $\mu$ g/ml) of fluorescein-conjugated goat ant-mouse IgG secondary antibody 20 (GIBCO BRL, Gaithersburg, MD) for 30 min. at 4°C in the dark. Following washing in binding buffer, the cells were analyzed using FACScan flow cytometer (Becton Dickinson). The gates were set on the live cell population, and the degree of inhibition of TNF- $\alpha$ /cell binding by peptides was calculated 25 on the basis of mean values of each histogram.

#### 6.1.6. CYTOTOXICITY ASSAY

A murine fibroblast cell line, L929, was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 30 and the medium was replaced with serum free AIM-V medium (GIBCO BRL) immediately before the cells were used for the cytotoxicity assay. L929 cells were seeded at a density of  $2 \times 10^5$  cells/ml in 96-well microtiter plates, and incubated for 20 hr. at 37°C under 5% CO<sub>2</sub> in air. TNF- $\alpha$  (7 pg) was 35 preincubated with a peptide solution (80  $\mu$ l) in PBS for 1 hr. at 37°C and was added to the cells which had been incubated with actinomycin D (ACT-D) at 1  $\mu$ g/ml for 2 hr. The cells



were incubated with TNF- $\alpha$  adjusted to a final concentration of 50 pg/ml for 7 hr. at 37°C under 5% CO<sub>2</sub>, and stained with MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide) (Sigma) (Hansen et al., 1989, J. Immunol. Methods 5 119:203). Briefly, 10  $\mu$ l of a 10 mg/ml solution of MTT were added to each well for 2 hr. at 37°C, and the color of the formazan product was developed by an overnight incubation at 37°C with 100  $\mu$ l of extraction buffer (20% SDS in 50% DMF, pH 4.7). The optical density of colored formazan was measured 10 at 600 nm.

#### 6.1.7. PHOSPHORYLATION OF IRS-1 IN ADIPOCYTES

3T3-L1 cells were grown and differentiated into adipocytes as described previously (Garcia de Herreros et 15 al., 1989, J. Biol. Chem. 264:19994). After maximal differentiation, (at least 90% of cells differentiated) the cells were starved in 0.2% BSA for 1 day and then treated with TNF- $\alpha$  at a concentration of 50 ng/ml in Leibovitz's L-15 medium/PBS (2 ml/ 2 ml) for 4 hr. at 37 C without CO<sub>2</sub>. In 20 certain samples, peptides were preincubated with TNF- $\alpha$  in PBS for 1 hr. at 37°C before the mixture was added to the starved adipocytes. The cells were then stimulated for 3 min. with insulin (100 nM), washed in ice-cold PBS, and then solubilized in lysis buffer (50 mM Hepes, pH 7.4, 150 mM 25 NaCl, 10 mM EDTA, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1mM Na<sub>3</sub>VO<sub>4</sub>, 100 mM NaF, 1% Triton X-100 (v/v), 10 $\mu$ g/ml aprotinin, 1 $\mu$ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). IRS-1 was immunoprecipitated from the cell extracts (1 ml) with an anti-IRS-1 antiserum (5 $\mu$ l, overnight at 4°C) and protein A- 30 Sepharose (Sigma) at 20  $\mu$ l bed volume for 1 hr. at 4°C. After three washes in 50 mM Hepes buffer (pH 7.4) including 1% Triton X-100 (v/v), 0.1% SDS and 150 mM NaCl, the immunoprecipitated proteins were boiled in Laemmli sample buffer for 3 min., and separated on 6% SDS-PAGE. Proteins 35 were then transferred to a nitrocellulose membrane, and Western blot analysis was performed using the ECL Western blot kit (Amersham) according to the manufacturer's

instructions. The membrane was first probed with an anti-phosphorylated Tyr antibody (1:20,000) coupled with a secondary labeled antibody, then stripped and reprobed with an anti-IRS-1 antiserum (1:1,000). The intensity of each  
5 band was analyzed by Image Quanta densitometer (Molecular Dynamics, Sunnyvale, CA), and the reduction of TNF- $\alpha$ -induced insulin resistance by peptides was calculated after subtracting background intensity from each band.

10

## 6.2. RESULTS

Amino acid sequences corresponding to three TNF- $\alpha$ -binding loops of TNF-R were used as templates for the synthesis of a number of peptides. Cys residues were included in the linear peptides to enable their cyclization.  
15 Their identity was verified by mass spectrometry.

Various exocyclic peptides listed in Table 2 were tested for their ability to inhibit radiolabeled TNF- $\alpha$  binding to TNF-R-chimeric protein. Figure 2 shows that while peptides designed from three separate loop regions of TNF-R inhibited  
20 TNF- $\alpha$  binding to TNF-R, peptides designed from loop 1 of domain 3 (WP9 series) of TNF-R exhibited stronger inhibitory effects than did the peptides designed from loop 1 of domain 2 (WP5 series) and loop 2 of domain 2 (WP8 series) at a concentration of 25  $\mu$ M. The WP5 series of peptides were  
25 developed by comparing the structures of TNF-R loops and CDR1 of an anti-TNF- $\alpha$  antibody. Modifications of the WP5 peptides from Ala to Asn increased their inhibitory activities, but similar modifications of the WP8 peptides did not result in such effects.

30 Loop 1 of domain 3 of TNF-R was used as a template to design a peptide which contained Gln instead of a charged residue, Glu (WP9Q). When the WP9Q peptide was tested in the competitive radioreceptor assay, it showed a 2-fold higher activity than the best WP5 peptides. Further modified WP9  
35 peptides were compared in a dose-response study, and the WP9QY peptide was shown to exhibit the strongest inhibitory activities, with an IC<sub>50</sub> at 5 $\mu$ M (Figure 3). The WP9QY

peptide was modified from WP9Q by replacing Asn in WP9Q with Tyr which enhanced receptor interactions with TNF- $\alpha$  via Trp and Gln which are critical residues for its binding to the receptor. A more compact shape of WP9QY than the other WP  
5 peptides also appeared to increase WP9QY interactions with TNF- $\alpha$  as suggested by its slower elution from a size exclusion column on HPLC (Figure 4).

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**Table 2**

Amino Acid Sequences of TNF- $\alpha$  Binding Sites in TNF-R and Exocyclic Peptides Derived from These Sites.

	TNF- $\alpha$ Binding Sites in the Receptor	Exocyclic Peptides*			
5	Binding site - 5				
	TNF- $\alpha$	WP5	YC	FTASENH	CY
	53E 82R 85VSY87 125Q 127E	WP5N	YC	FTNSENH	CY
		WP5R	YC	FTRSENH	CY
	Receptor (loop 1 of domain2)	WP5J	FC	ASENH	CY
10	60 FTASENH 66	WP5JY	YC	ASENH	CY
		WP5JN	FC	NSENH	CY
		WP5JR	FC	NSENR	CY
		WP5VR	FC	NSVENR	CY
		WP1**	YC	SQSVSND	CF
		WP1R**	FC	VSNDR	CY
15	Binding site - 8				
	TNF- $\alpha$				
	65K 67Q 113P 115Y 143L 145A				
		WP8L	YC	RKELGQV	CY
	Receptor (loop 2 of domain 2)	WP8JP	YC	KEPGQ	CY
20	76 CRKEMGQV 83	WP8J	YC	RKEMG	CY
		WP8JF	FC	RKEMG	CY
	Binding site - 9				
	TNF- $\alpha$				
	72THVL75 77T 97I 137 N				
25		WP9Q	YC	WSQNL	CY
	Receptor (loop 1 of domain3)	WP9ELY	YCELSQYL		CY
	107 WSENL 111	WP9Y	YC	WSQNY	CY
		WP9QY	YC	WSQYL	CY
30	* Peptides were cyclized with cysteine disulfide bridges				
	** WP1 and WP1R were derived from an anti-TNF- $\alpha$ antibody (Di62, CDR1L), and the template sequence is QSVSNDV.				

U937 cells which express high levels of TNF-R p55 were incubated with TNF- $\alpha$  with or without peptides. The binding of TNF- $\alpha$  to the cells was quantitated by an anti-TNF-R antibody followed by FACS analysis. Figure 5 demonstrates that while significant binding of anti-receptor antibody to

U937 cells was observed, there was a 70% reduction of this binding by pretreatment of the cells with TNF- $\alpha$ . The addition of the WP9QY peptide caused anti-receptor staining to return to a level similar to that with anti-receptor  
5 antibody alone. When a similar experiment was performed in a dose-response assay, the WP9QY peptide again exhibited the strongest inhibitory activity as compared to WP9Q and WP5JY peptides (Figure 6). The WP9QY peptide inhibited TNF- $\alpha$  binding to its cellular receptor with an IC<sub>50</sub> at 75  $\mu$ M.

10 Additionally, the peptides were tested for their ability to inhibit the biological effects induced by TNF- $\alpha$  on TNF-sensitive L929 cells (Hennet et al., 1993, Biochem. J. 289:587-592). TNF- $\alpha$  induced apoptosis/cytotoxicity of L929 cells, and the WP9QY peptide protected nearly 90% of the  
15 cells against such effects of TNF- $\alpha$  at a concentration of 75  $\mu$ M (Figure 7). WP9Q also showed dose-dependent inhibitory activity which corresponded to about half of the effects of WP9QY.

Previous studies have shown that 3T3-L1 fibroblastic  
20 cell line undergoes differentiation to adipocytes under appropriate culture conditions, and the differentiated cells develop an increased sensitivity to insulin (Garcia de Herreros et al., 1989, J. Biol. Chem. 264:19994). Response to insulin stimulation in these cells can be conveniently  
25 measured by tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1). The addition of TNF- $\alpha$  has been shown to diminish insulin-induced phosphorylation of this substrate, suggesting that TNF- $\alpha$  may play a role in obesity-induced insulin resistance (Kanety et al., 1995, J. Biol. Chem.  
30 270:23780).

The WP9QY peptide was tested for its ability to inhibit TNF- $\alpha$ -induced-insulin resistance. 3T3-L1 cells were induced to differentiate into adipocytes, stimulated with insulin and Tyr phosphorylation of IRS-1 was assayed by  
35 immunoprecipitating the cell extracts with anti-IRS-1 antiserum followed by Western blot analysis with an anti-phosphorylated Tyr antibody. Figure 8A shows that

phosphorylated IRS-1 was detected in cells stimulated with 100 nM insulin. In contrast, when TNF- $\alpha$  (50 ng/ml) was added to the cells prior to insulin, the same IRS-1 band was not detectable, confirming that TNF- $\alpha$  inhibited insulin

5 stimulation of Tyr phosphorylation of IRS-1. However, when the WP9QY peptide was preincubated with TNF- $\alpha$ , it antagonized the effects of TNF- $\alpha$  in a dose-dependent manner, as evidenced by the reappearance of the phosphorylated IRS-1 band as the concentrations of the peptide increased. Figure 8B  
10 demonstrates that IRS-1 was present in all tested samples.

In conclusion, peptides derived from certain loop structures of TNF-R inhibit the binding of TNF- $\alpha$  to cell surface TNF-R, indicating that these loops correspond to binding sites of TNF-R to its ligands. The peptides also  
15 antagonize the biological effects of TNF- $\alpha$  in preventing the induction of apoptosis/cytotoxicity and insulin resistance in target cells. Importantly, the most effective peptide is derived from loop 1 of domain 3 of TNF-R, which may represent a critical binding site between TNF-R and TNF- $\alpha$  interactions.  
20 Figure 9 presents a three-dimensional model of TNF- $\alpha$  (right side) and TNF-R (left side) interactions. The darkened portion of the loop structure in TNF-R represents the location of loop 1 of domain 3.

The present invention is not to be limited in scope by  
25 the exemplified embodiments which are intended as illustrations of single aspects of the invention and any sequences which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those shown and described herein  
30 will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All publications cited herein are incorporated by  
35 reference in their entirety.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: Greene, Mark I.  
Murali, Ramachandran  
Takasaki, Wataru
- (ii) TITLE OF THE INVENTION: PEPTIDES AND PEPTIDE  
ANALOGUES DESIGNED FROM BINDING SITES OF TUMOR  
NECROSIS FACTOR RECEPTOR SUPERFAMILY AND THEIR  
USES
- (iii) NUMBER OF SEQUENCES: 27
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Pennie & Edmonds LLP
  - (B) STREET: 1155 Avenue of the Americas
  - (C) CITY: New York
  - (D) STATE: NY
  - (E) COUNTRY: USA
  - (F) ZIP: 10036-2811
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: DOS
  - (D) SOFTWARE: FastSEQ Version 2.0
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/866,545
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- (vii) PRIOR APPLICATION DATA:
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- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Coruzzi, Laura A
  - (B) REGISTRATION NUMBER: 30,742
  - (C) REFERENCE/DOCKET NUMBER: 009113-0004-999
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 650-493-4935
  - (B) TELEFAX: 650-493-5556
  - (C) TELEX: 66141 PENNIE

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 74 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: None
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His  
 1 5 10 15

Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln  
                   20                  25                  30  
 Val Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys  
                   35                  40                  45  
 Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys  
           50                  55                  60  
 Phe Asn Cys Ser Leu Cys Leu Asn Gly Thr  
 65                  70

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 77 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu Trp Asn Trp  
   1                  5                  10                  15  
 Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser Asp Gln Val  
                   20                  25                  30  
 Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys Thr Cys Arg  
                   35                  40                  45  
 Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys Arg Leu Cys  
           50                  55                  60  
 Ala Pro Leu Arg Lys Cys Arg Pro Gly Phe Gly Val Ala  
 65                  70                  75

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 77 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Val Cys Ala Thr Cys Ala Glu Asn Ser Tyr Asn Glu His Trp Asn Tyr  
   1                  5                  10                  15  
 Leu Thr Ile Cys Gln Leu Cys Arg Pro Cys Asp Pro Val Met Gly Leu  
                   20                  25                  30  
 Glu Glu Ile Ala Pro Cys Thr Ser Lys Arg Lys Thr Gln Cys Arg Cys  
                   35                  40                  45  
 Gln Pro Gly Met Phe Cys Ala Ala Trp Ala Leu Glu Cys Thr His Cys  
           50                  55                  60  
 Glu Leu Leu Ser Asp Cys Pro Pro Gly Thr Glu Ala Glu  
 65                  70                  75

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 76 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:



```

Cys Glu Pro Cys Leu Asp Ser Val Thr Phe Ser Asp Val Val Ser Ala
 1           5           10           15
Thr Glu Pro Cys Lys Pro Cys Thr Glu Cys Val Gly Leu Gln Ser Met
           20           25           30
Ser Ala Pro Cys Val Glu Ala Asp Asp Ala Val Cys Arg Cys Ala Tyr
           35           40           45
Gly Tyr Thr Gln Asp Glu Thr Thr Gly Arg Cys Glu Ala Cys Arg Val
 50           55           60
Cys Glu Ala Gly Ser Gly Leu Val Phe Ser Cys Gln
65           70           75

```

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Gln Cys Asp Pro Cys Ile Pro Gly Val Ser Phe Ser Pro Asp His His
 1           5           10           15
Thr Arg Pro His Cys Glu Ser Cys Arg His Cys Asn Ser Gly Leu Leu
           20           25           30
Val Arg Asn Cys Thr Ile Thr Ala Asn Ala Glu Cys Ala Cys Arg Asn
           35           40           45
Gly Trp Gln Cys Arg
50

```

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 74 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Cys Arg Lys Gln Cys Glu Pro Asp Tyr Tyr Leu Asp Glu Ala Asp Arg
 1           5           10           15
Cys Thr Ala Cys Val Thr Cys Ser Arg Asp Asp Leu Val Glu Lys Thr
           20           25           30
Pro Cys Ala Trp Asn Ser Ser Arg Val Cys Glu Cys Arg Pro Gly Met
           35           40           45
Phe Cys Ser Thr Ser Ala Val Asn Ser Cys Ala Arg Cys Phe Phe His
 50           55           60
Ser Val Cys Pro Ala Gly Met Ile Val Lys
65           70

```

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 74 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

Cys Arg Lys Gln Cys Glu Pro Asp Tyr Tyr Leu Asp Glu Ala Gly Arg
 1      5      10
Cys Thr Ala Cys Val Ser Cys Ser Arg Asp Asp Leu Val Glu Lys Thr
      20      25      30
Pro Cys Ala Trp Asn Ser Ser Arg Thr Cys Glu Cys Arg Pro Gly Met
      35      40      45
Ile Cys Ala Thr Ser Ala Thr Asn Ser Cys Ala Arg Cys Val Pro Tyr
 50      55      60
Pro Ile Cys Ala Ala Glu Thr Val Thr Lys
65      70

```

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 75 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

Glu Cys Leu Pro Cys Gly Glu Ser Glu Phe Leu Asp Thr Trp Asn Arg
 1      5      10
Glu Thr His Cys His Gln His Lys Tyr Cys Asp Pro Asn Leu Gly Leu
      20      25      30
Arg Val Gln Gln Lys Gly Thr Ser Glu Thr Asp Thr Ile Cys Thr Cys
      35      40      45
Glu Glu Gly Trp His Cys Thr Ser Glu Ala Cys Glu Ser Cys Val Leu
 50      55      60
His Arg Ser Cys Ser Pro Gly Phe Gly Val Lys
65      70      75

```

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

Asp Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His
 1      5      10
Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Glu Gly His Gly
      20      25      30
Leu Glu

```

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 56 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```

Cys Arg Pro Cys Gly Pro Gly Phe Tyr Asn Asp Val Val Ser Ser Lys
 1      5      10      15

```

```

Pro Cys Lys Pro Cys Thr Trp Cys Asn Leu Arg Ser Gly Ser Glu Arg
      20      25      30
Lys Gln Leu Cys Thr Ala Thr Gln Asp Thr Val Cys Arg Cys Arg Ala
      35      40      45
Gly Thr Gln Pro Leu Asp Ser Tyr
      50      55

```

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Cys Ser Pro Cys Pro Pro Asn Ser Phe Ser Ser Ala Gly Gly Gln Arg
 1      5      10      15
Thr Cys Asp Ile Cys Arg Gln Cys Lys Gly Val Phe Arg Thr Arg Lys
      20      25      30
Glu Cys Ser Ser Thr Ser Asn Ala Glu Cys Asp Cys Thr Pro Gly Phe
      35      40      45
His Cys Leu Gly Ala Gly Cys Ser Met Cys Glu Gln Asp Cys Lys Gln
      50      55      60
Gly Gln Glu Leu Thr
65

```

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Tyr Cys Glu Leu Ser Gln Tyr Leu Cys Tyr
 1      5      10

```

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

Tyr Cys Trp Ser Gln Asn Leu Cys Tyr
 1      5

```

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Tyr Cys Trp Ser Gln Asn Tyr Cys Tyr  
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Tyr Cys Trp Ser Gln Tyr Leu Cys Tyr  
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Tyr Cys Phe Thr Ala Ser Glu Asn His Cys Tyr  
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Tyr Cys Phe Thr Asn Ser Glu Asn His Cys Tyr  
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Tyr Cys Phe Thr Arg Ser Glu Asn His Cys Tyr  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Phe Cys Ala Ser Glu Asn His Cys Tyr  
1 5

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Tyr Cys Ala Ser Glu Asn His Cys Tyr  
1 5

## (2) INFORMATION FOR SEQ ID NO:21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Phe Cys Asn Ser Glu Asn His Cys Tyr  
1 5

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Phe Cys Asn Ser Glu Asn Arg Cys Tyr  
1 5

## (2) INFORMATION FOR SEQ ID NO:23:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Phe Cys Asn Ser Val Glu Asn Arg Cys Tyr  
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Tyr Cys Arg Lys Glu Leu Gly Gln Val Cys Tyr  
1 5 10

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Tyr Cys Lys Glu Pro Gly Gln Cys Tyr  
1 5

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Tyr Cys Arg Lys Glu Met Gly Cys Tyr  
1 5

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Phe Cys Arg Lys Glu Met Gly Cys Tyr  
1 5

WHAT IS CLAIMED IS:

1. A compound having the formula:



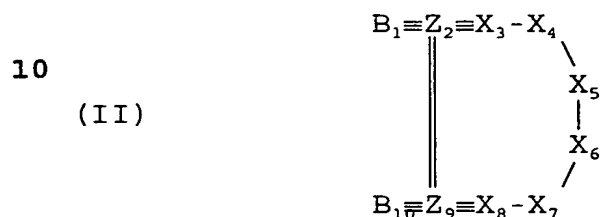
wherein:

- 10 AC is a peptide of 3-18 amino acid residues which corresponds in primary sequence to a binding loop of a TNF-R superfamily member, and which may optionally contain one or more amino acid substitutions, or an analogue thereof wherein at least one amide linkage is replaced with a substituted amide or an isostere of amide;
- 15 AB<sub>1</sub> is a moiety having a first functional group capable of forming a covalent linkage with one terminus of AC, a second functional group capable of forming a covalent linkage with AB<sub>2</sub> and a third functional group capable of forming a covalent linkage with AA<sub>1</sub>;
- 20 AB<sub>2</sub> is a moiety having a first functional group capable of forming a covalent linkage with the second terminus of AC, a second functional group capable of forming a covalent linkage with AB<sub>1</sub> and a third functional group capable of forming a covalent linkage with AA<sub>2</sub>;
- 25 AA<sub>1</sub> is a moiety having hydrophobic properties and a functional group capable of forming a covalent linkage with the third functional group of AB<sub>1</sub>;
- 30 AA<sub>2</sub> is a moiety having hydrophobic properties and a functional group capable of forming a covalent linkage with the third functional group of AB<sub>2</sub>;
- "=" is a covalent linkage; and
- 35 "≡" is a covalent linkage.

2. The compound of Claim 1 in which the amino acid substitutions are conservative.

3. The compound of Claim 2 in which the member of TNF-R superfamily is TNF-R p55.

4. The compound of Claim 3, which has the formula:



15 wherein:

$B_1$  and  $B_{10}$  are each independently a peptide of 1-6 amino acids, at least one of which is a hydrophobic amino acid, an aromatic moiety or a heteroaromatic moiety;

20  $Z_2$  is a moiety that is capable of forming a covalent linkage with  $B_1$ ,  $X_3$  and  $Z_9$ ;

$Z_9$  is a moiety that is capable of forming a covalent linkage with  $B_{10}$ ,  $X_8$  and  $Z_2$ ;

$X_3$  is absent or a hydrophilic amino acid;

25  $X_4$  is a hydrophobic amino acid;

$X_5$  is a hydrophilic amino acid;

$X_6$  is a hydrophilic amino acid;

$X_7$  is a hydrophobic or hydrophilic amino acid;

$X_8$  is a hydrophobic or hydrophilic amino acid;

30 "-" is an amide, substituted amide or an isostere of amide thereof;

"=" is a covalent linkage; and

"≡" is a covalent linkage.

5. The compound of Claim 4, wherein:

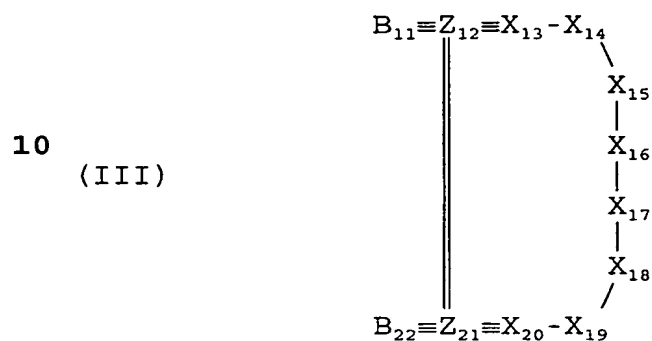
35  $B_1$  and  $B_{10}$  are each independently a peptide of 1-3 amino acids, at least one of which is an aromatic amino acid;



- Z<sub>2</sub> and Z<sub>9</sub> are each independently a Cys-like amino acid;  
X<sub>3</sub> is absent or an acidic amino acid;  
X<sub>4</sub> is an aromatic or apolar amino acid;  
X<sub>5</sub> is a polar amino acid;  
5 X<sub>6</sub> is a polar amino acid;  
X<sub>7</sub> is an aromatic or polar amino acid;  
X<sub>8</sub> is an aromatic, apolar or polar amino acid;  
"-" is an amide linkage;  
"=" is a disulfide linkage; and  
10 "≡" is an amide linkage.
6. The compound of Claim 5, wherein:  
B<sub>1</sub> and B<sub>10</sub> are each independently Tyr or Phe;  
Z<sub>2</sub> and Z<sub>9</sub> are each Cys;  
15 X<sub>3</sub> is absent or Glu;  
X<sub>4</sub> is Trp or Leu;  
X<sub>5</sub> is Ser;  
X<sub>6</sub> is Gln;  
X<sub>7</sub> is Tyr or Asn;  
20 X<sub>8</sub> is Tyr or Leu;  
"-" is an amide linkage;  
"=" is a disulfide linkage; and  
"≡" is an amide linkage.
- 25 7. The compound of Claim 6, wherein said compound is selected from the group consisting of WP9Q (SEQ ID NO:13), WP9ELY (SEQ ID NO:12), WP9Y (SEQ ID NO:14) and WP9QY (SEQ ID NO:15).
- 30 8. A pharmaceutical composition, comprising the compound of Claim 4 and a pharmaceutical excipient carrier or an excipient.
9. A method of inhibiting TNF binding to a receptor,  
35 comprising administering to a subject a therapeutically effective amount of the compound of Claim 4.

10. A method of treating a TNF-associated pathological condition, comprising administering to a subject a therapeutically effective amount of the compound of Claim 4.

5 11. The compound of Claim 3, which has the formula:



15 wherein:

$B_{11}$  and  $B_{22}$  are each independently a peptide of 1-6 amino acids, at least one of which is a hydrophobic amino acid, an aromatic moiety or a heteroaromatic moiety;

20  $Z_{12}$  is a moiety that is capable of forming a covalent linkage with  $B_{11}$ ,  $X_{13}$  and  $Z_{21}$ ;

$Z_{21}$  is a moiety that is capable of forming a covalent linkage with  $B_{22}$ ,  $X_{20}$  and  $Z_{12}$ ;

$X_{13}$  is absent or hydrophobic amino acid;

25  $X_{14}$  is absent or a hydrophilic amino acid;

$X_{15}$  is a hydrophilic or hydrophobic amino acid;

$X_{16}$  is a hydrophilic amino acid;

$X_{17}$  is absent or a hydrophobic amino acid;

$X_{18}$  is a hydrophilic amino acid;

30  $X_{19}$  is a hydrophilic amino acid;

$X_{20}$  is a hydrophilic amino acid;

"-" is an amide, a substituted amide or an isostere of amide thereof;

"=" is a covalent linkage; and

35 "≡" is a covalent linkage.

12. The compound of Claim 11, wherein:
- B<sub>11</sub> and B<sub>22</sub> are each independently a peptide of 1-3 amino acids, at least one of which is an aromatic amino acid;
- Z<sub>12</sub> and Z<sub>21</sub> are each independently a Cys-like amino acid;
- 5 X<sub>13</sub> is absent or an aromatic amino acid;
- X<sub>14</sub> is absent or a polar amino acid;
- X<sub>15</sub> is a basic, polar or apolar amino acid;
- X<sub>16</sub> is a polar amino acid;
- X<sub>17</sub> is absent or an apolar amino acid ;
- 10 X<sub>18</sub> is an acidic amino acid;
- X<sub>19</sub> is a polar amino acid;
- X<sub>20</sub> is a basic amino acid;
- "-" is an amide linkage;
- "=" is a disulfide linkage; and
- 15 "≡" is an amide linkage.

13. The compound of Claim 12, wherein:
- B<sub>11</sub> and B<sub>22</sub> are each independently Tyr or Phe;
- Z<sub>12</sub> and Z<sub>21</sub> are each Cys;
- 20 X<sub>13</sub> is absent or Phe;
- X<sub>14</sub> is absent or Thr;
- X<sub>15</sub> is Ala, Asn or Arg;
- X<sub>16</sub> is Ser;
- X<sub>17</sub> is absent or Val;
- 25 X<sub>18</sub> is Glu;
- X<sub>19</sub> is Asn;
- X<sub>20</sub> is Arg or His;
- "-" is an amide linkage;
- "=" is a disulfide linkage; and
- 30 "≡" is an amide linkage.

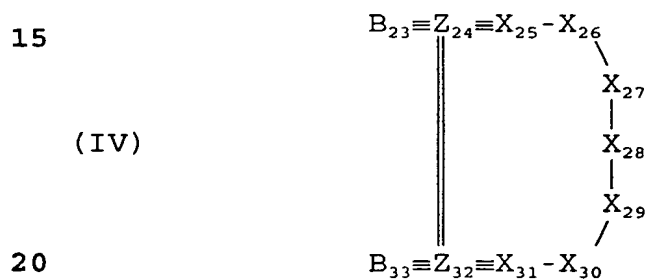
14. The compound of Claim 13, wherein said compound is selected from the group consisting of WP5 (SEQ ID NO:16), WP5N (SEQ ID NO:17), WP5R (SEQ ID NO:18), WP5J (SEQ ID NO:19), WP5JY (SEQ ID NO:20), WP5JN (SEQ ID NO:21), WP5JR (SEQ ID NO:22) and WP5VR (SEQ ID NO:23).

15. A pharmaceutical composition, comprising the compound of Claim 11 and a pharmaceutical excipient carrier or an excipient.

5 16. A method of inhibiting TNF binding to a receptor, comprising administering to a subject a therapeutically effective amount of the compound of Claim 11.

17. A method of treating a TNF-associated pathological  
10 condition, comprising administering to a subject a therapeutically effective amount of the compound of Claim 11.

18. The compound of Claim 3, which has the formula:



wherein:

$B_{23}$  and  $B_{33}$  are each independently a a peptide of 1-6 amino acids, at least one of which is a hydrophobic amino  
25 acid, an aromatic moiety or a heteroaromatic moiety;

$Z_{24}$  is a moiety that is capable of forming a covalent linkage with  $B_{23}$ ,  $X_{25}$  and  $Z_{32}$ ;

$Z_{32}$  is a moiety that is capable of forming a covalent  
30 linkage with  $B_{33}$ ,  $X_{31}$  and  $Z_{24}$ ;

$X_{25}$  is absent or a hydrophilic amino acid;

$X_{26}$  is a hydrophilic amino acid;

$X_{27}$  is a hydrophilic amino acid;

$X_{28}$  is a hydrophobic amino acid;

$X_{29}$  is a hydrophobic amino acid;

35  $X_{30}$  is absent or a hydrophilic amino acid;

$X_{31}$  is absent or a hydrophobic amino acid;

"-" is an amide, a substituted amide or an isostere of amide;

"=" is a covalent linkage; and

"≡" is a covalent linkage.

5

19. The compound of Claim 18, wherein:

B<sub>23</sub> and B<sub>33</sub> are each independently a peptide of 1-3 amino acids, at least one of which is an aromatic amino acid;

Z<sub>24</sub> and Z<sub>32</sub> are each independently a Cys-like amino acid;

10 X<sub>25</sub> is absent or a basic amino acid;

X<sub>26</sub> is a basic amino acid;

X<sub>27</sub> is an acidic amino acid;

X<sub>28</sub> is an apolar amino acid;

X<sub>29</sub> is an apolar amino acid;

15 X<sub>30</sub> is absent or a polar amino acid;

X<sub>31</sub> is absent or an apolar amino acid;

"-" is an amide linkage;

"=" is a disulfide linkage; and

"≡" is an amide linkage.

20

20. The compound of Claim 19, wherein:

B<sub>23</sub> and B<sub>33</sub> are each independently Tyr or Phe;

Z<sub>24</sub> and Z<sub>32</sub> are each Cys;

X<sub>25</sub> is absent or Arg;

25 X<sub>26</sub> is Lys;

X<sub>27</sub> is Glu;

X<sub>28</sub> is Leu, Pro or Met;

X<sub>29</sub> is Gly;

X<sub>30</sub> is absent or Gln;

30 X<sub>31</sub> is absent or Val;

"-" is an amide linkage;

"=" is a disulfide linkage; and

"≡" is an amide linkage.

35 21. The compound of Claim 20, wherein said compound is selected from the group consisting of WP8L (SEQ ID NO:24),

WP8JP (SEQ ID NO:25), WP8J (SEQ ID NO:26) and WP8JF (SEQ ID NO:27) .

22. A pharmaceutical composition, comprising the compound of  
5 Claim 18 and a pharmaceutical excipient carrier or an excipient.

23. A method of inhibiting TNF binding to a receptor,  
comprising administering to a subject a therapeutically  
10 effective amount of the compound of Claim 18.

24. A method of treating a TNF-associated pathological  
condition, comprising administering to a subject a  
therapeutically effective amount of the compound of Claim 18.  
15

25. A method of selecting an agent which antagonizes TNF  
activities, comprising incubating TNF with the compound of  
Claim 1 in the presence of a test agent, and measuring a  
decrease in TNF binding to the compound.  
20

25

30

35

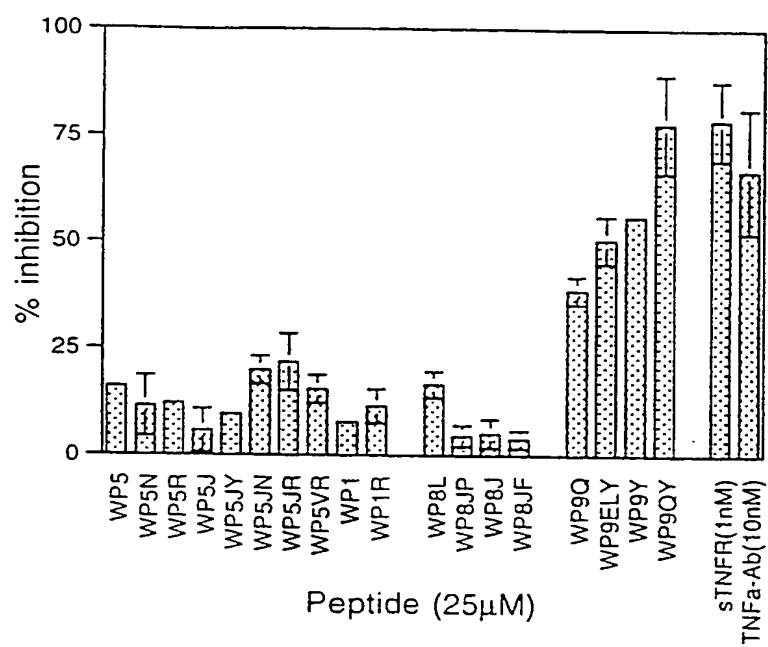
	70	80	90	100	110	120	130	140
<i>TNF-R p55</i>	DCRECEGSFTASENHLRHCLSCSK.. CRKEMGQVEISS	CTVDRDTV CGCRKNQYRHYWSENLFQ. C	FNCSLCLNG... T					
<i>TNF-R p75</i>	VCDSCEDSTYTQLWNWVPECLSCGSRG SSDQVE...TQA	CTREQNRI CTCRPGWYCALSKQEGCRL C	APLRKCRPGFGV A					
<i>TNF-R rp</i>	VCATCAENSYNHWNLYL..TICQLCRP CDPVMGLEEIAP	CTSKRKTQ CRCQPGMFCAAWALE.CTH C	ELLSDCPPGTEA E					
<i>NGF-R p75</i>	.CEPCLDSVTFSDVVSATEPKPCTEC VGLQSMSAP...	CVEADDAV CRCAYGYVQDETTCRCEAC R	VC EAGS GLVFSC Q					
<i>CD27</i>	QCDCPCIPGVFSFS...PDHHTRPHCESC RHCNSGLLLVRN.	CTITANAE CACRNGW.....	.....QC R					
<i>CD30(proximal)</i>	CRKQCEPDYVLDEADRCTACVTCSR.D. ....DLVEKTP	CAWNSSRV CECRPGMFCSTSAVNScar C	FFHSVC PAGMIV K					
<i>CD30(distal)</i>	CRKQCEPDYVLDEAGRCTACVSCSRD. ....DLVEKTP	CAWNSSRT CECRPGMICATSATNSCAR C	VPYPICAAETVT K					
<i>CD40</i>	ECLPCGESEFELDTWNR..ETHCHQHKY CDPNLGLRVQOK GTSETDTI	CTCEEGWHT...SEACES C	VLHRSC SPGFGV K					
<i>Fas</i>	DCVPCQEGKEYTDKAHF.....SSK..	CRR.....	...CRLCDEGHGL E					
<i>OX40</i>	.CRPC.GPGFYNDVVSS.KPCKPCTWC .NLRSGSERKQL	CTATQDTV CR.....	.CRAGTQPLDSY .					
<i>4-1BB</i>	.CSPCPPNSF.....SSAGGQRTCDIC RQCKGVFRTRKE	CSSTSNAE CDCTPGFH.....CLGAGC S MC	EQDCKQGGEL T					

Figure 1

9113-004 (Sheet 1 of 9)

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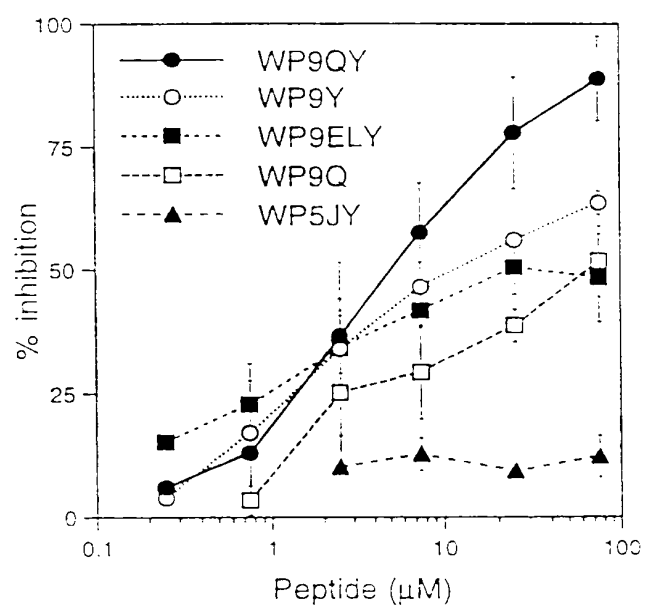
Figure 2





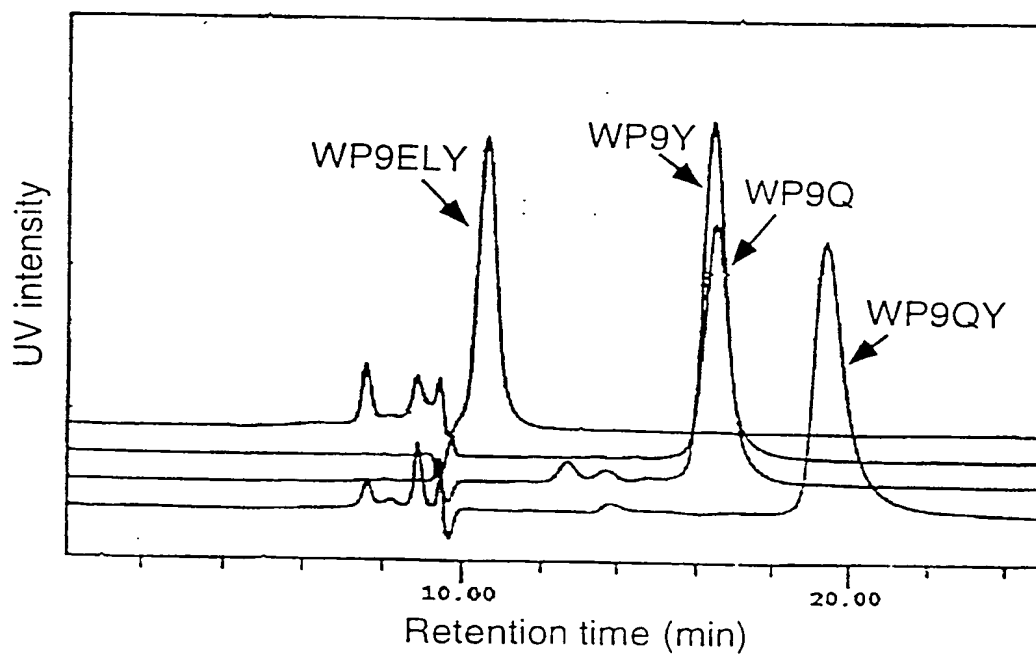
9113-004 (sheet 3 of 9)

Figure 3



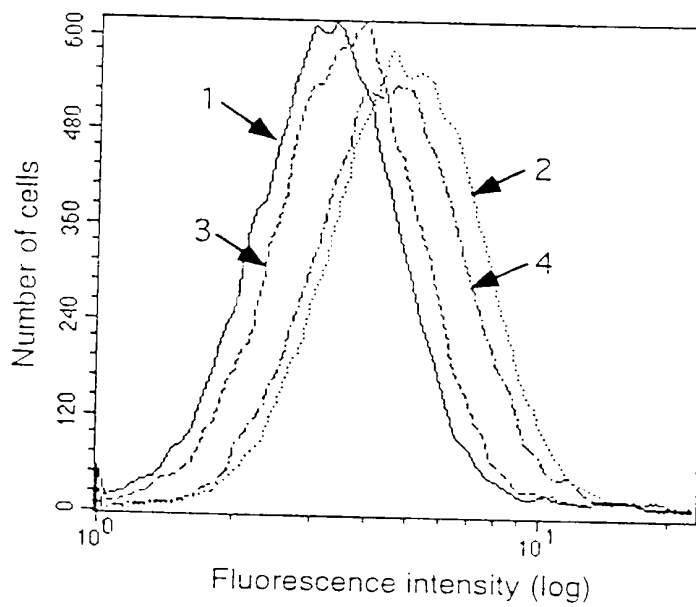
9113-004 (Sheet 4 of 9)

Figure 4



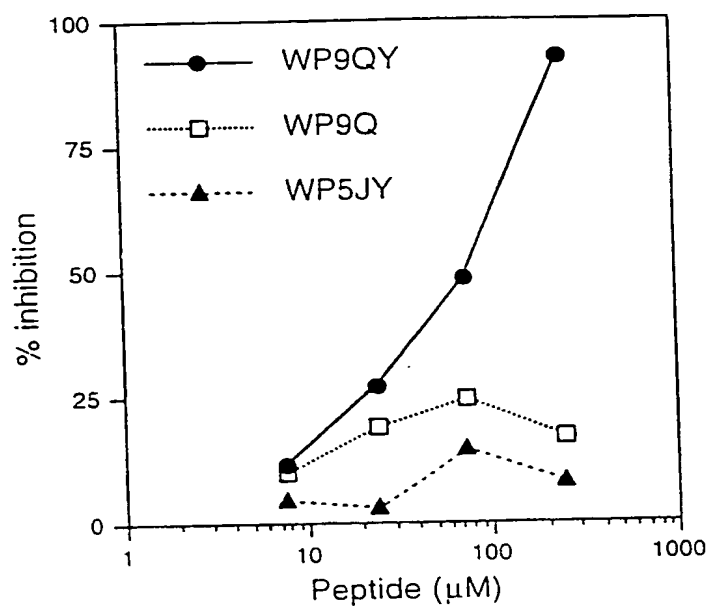
9113-004 (Sheet 5 of 9)

Figure 5



9113-004 (Sheet 6 of 9)

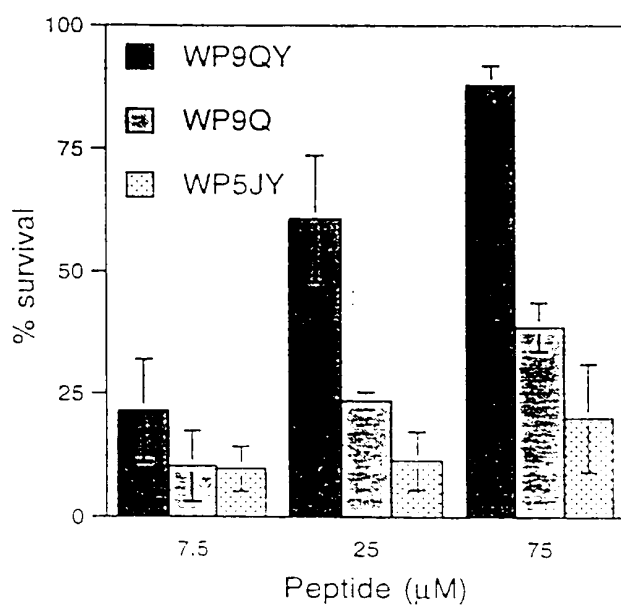
Figure 5



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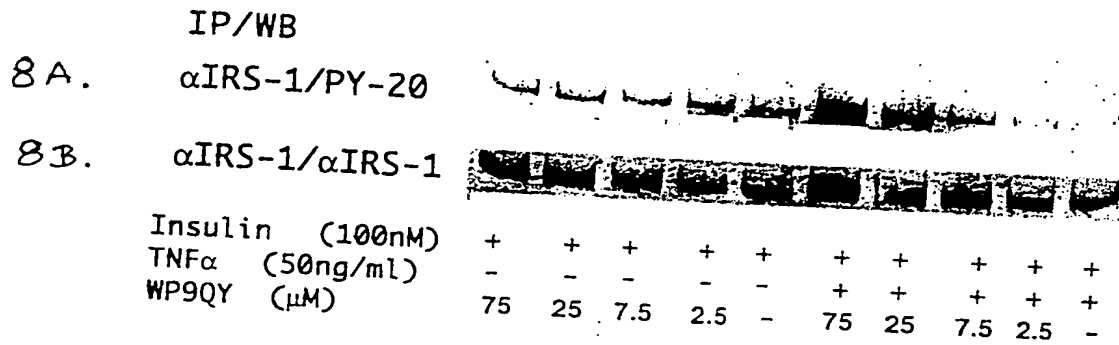
9113-004 (Sheet 7 of 9)

Figure 7



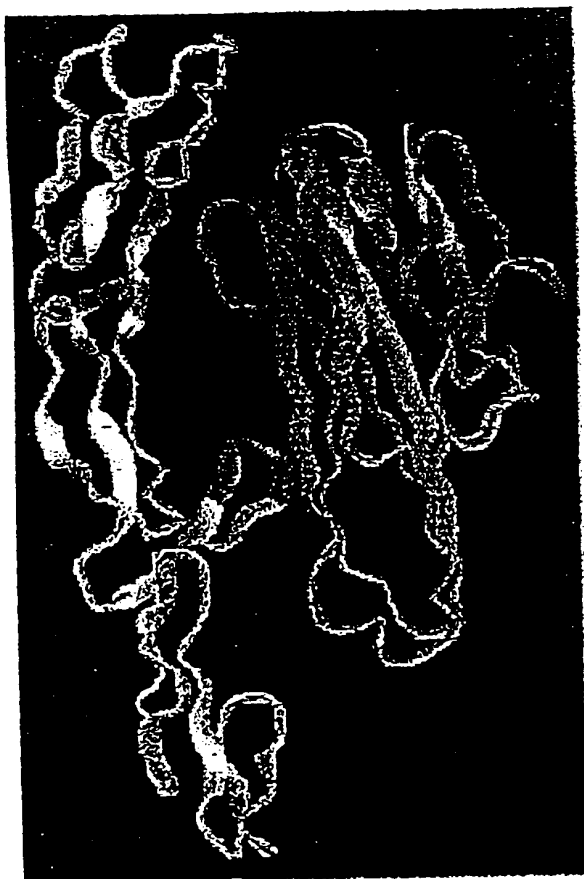
9113-004 (sheet 8 of 9)

Figure 8 A and 8 B



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Figure 9



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/10891**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 38/00; C07K 7/50, 7/64

US CL :514/11; 530/321

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/11; 530/321

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN/MEDLINE

search terms: cyclic peptide#, tumor necrosis factor#, TNF#, receptor#, CD27, CD30, CD40,

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — A	BANNER ET AL., Crystal Structure of the Soluble Human 55kd TNF Receptor-Human TNF beta Complex: Implications for TNF Receptor Activation. Cell. 07 May 1993, Vol. 73, pages 431-445, see Figure 1B.	1-3 ----- 4-25
A,P	NAISMITH ET AL., Modularity in the TNF-receptor family. Trends in Biochemical Sciences. February 1998, Vol. 23, No. 2, pages 74-79.	1-25

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

20 AUGUST 1998

Date of mailing of the international search report

24 SEP 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JOHN D. ULM

Telephone No. (703) 308-0196